ECOLE POLYTECHNIQUE FEDERALE DE LAUSANNE

SCHOOL OF LIFE SCIENCES

MASTER'S PROJECT IN BIOENGINEERING AND BIOTECHNOLOGY

Investigation of Integrin Expression in Human Mesenchymal Stem Cells during Chondrogenesis in Different Culture Systems

Carried out in the laboratory of Professor Molly M. Stevens
at Imperial College London,
under the supervision of Vanessa LaPointe, PhD student.

Done by

Amanda Verpoorte

Under the direction of Professor Jeffrey A. Hubbell

Institute of Bioengineering
SWISS FEDERAL INSTITUTE OF TECHNOLOGY –EPFL

External Expert : Dr Rekha Nair, Post doctoral researcher.

Lausanne, EPFL 2011

Acknowledgment

The completion of this project would not have been possible without the support of my colleagues, friends and family that I would like to acknowledge here.

First, I would like to thank Vanessa LaPointe who supervised me for this project and who helped me to become a qPCR Master. She has made her support available in numerous ways, this was highly appreciated and valued.

I would like to express my gratitude to Helene Autefage who always encouraged me and gave me countless advices to pursue the project. A special thank also to Matthew Hembury for his help at critical moments during this year in the laboratory.

I am indebted to many of my colleagues for their support and of course my office mates Rekha Nair and Lise de Jonge for answering every questions, scientific or not.

I would like to thank you also Professor Molly Stevens for giving me the opportunity to carry out my Master project in her laboratory at Imperial College London with extraordinary people. That was a one-time experience that I highly appreciate.

Of course, I am grateful to Professor Jeffrey Hubbell for helping me to carry out my Master thesis abroad and for his supervision from Switzerland.

Finally, last but not least I thank my mother, father and sister for their unconditional support and for never doubting that “I will make it”.

1. Abstract

Cartilage tissue engineering aims to develop a material that can be implanted with cells to repair damage in articular cartilage. The extracellular matrix is known to play a key role in regulating stem cell behaviour via cell-matrix interactions and integrins are major cell adhesion receptors that act as intermediates between cells and their surrounding matrix. Integrin-mediated signalling is known to be important in chondrocytes and to drive chondrogenesis. In our study, we investigated integrin expression during chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells (hMSCs) between three different high density 3D culture methods: pellet culture, micromass culture and collagen type II 3D hydrogels. Integrins are αβ heterodimeric transmembrane proteins, so we quantified the mRNA expression of every alpha and beta subunit using quantitative polymerase chain reaction over a period of 21 days. In all culture methods, alpha and beta integrin subunits were down-regulated in hMSCs undergoing chondrogenesis compared to the same cells in growth medium, with the exception for integrin subunits α11 and αV which were upregulated in cells undergoing chondrogenesis . Moreover, similar transcript expression was identified irrespective of the culture system for the alpha subunits related to collagen-binding integrins, while the expression of fibronectin- and laminin-binding receptors was greatly variable and dependent on the culture method. In conclusion, this work has led to an increased understanding of the expression of integrins in hMSCs and during chondrogenesis. This knowledge can be used to design materials that present the ligands for these integrins to further improve cartilage tissue engineering

Key words: cartilage tissue engineering, chondrogenesis, extracellular matrix, integrins, mesenchymal stem cells.

2. Introduction and Scientific Background

2.1. Project Aim and Motivation

Cartilage damages are common in daily life and is a major cause of disability as is osteoarthritis for elderly people. Yet cartilage tissue has little self-healing potential due to its avascular nature and the limited ability of chondrocytes to migrate and repair the damaged site, making cartilage injury a major clinical concern. Specifically, none of the existing treatments are entirely satisfactory, each having important drawbacks such as producing fibrocartilage, creating secondary morbid site or requiring major surgery.

Regenerative medicine is a promising alternative treatment that aims to meet clinical need for cartilage replacement. Cartilage tissue engineering is a multidisciplinary approach which involves combination of cells, scaffolds, and biological cues in order to recreate the tissue. Mesenchymal stem cells (MSCs) are a particularly promising cell source, as they can be easily isolated from the patient, and are capable of both self-renewal and chondrogenic differentiation. While there have been many promising advances in recent years, it remains a challenge to design a material that can promote firstly the early adhesion of MSCs, their subsequent chondrogenesis, and then support the fully differentiated chondrocytes.

For this reason, we chose to investigate the changing adhesion requirements of MSCs during chondrogenesis, with the intent to better inform the design of cartilage tissue engineering scaffolds. It is well established that the surrounding extracellular matrix regulates stem cell behaviour. These cell-matrix interactions are mostly mediated by integrins which thus have a central role in modulating stem cell differentiation. Integrins are a major surface adhesion receptor family composed of 24 distinct heterodimeric members interacting with several ligands found in cartilage including collagen types I, II, VI, IX, fibronectin, COMP, osteopontin, laminin, etc. We investigated mRNA integrin subunits expression during hMSCs chondrogenic differentiation in three different culture systems which are the gold standard pellet culture, micromass culture and collagen type II hydrogels. We seek to evaluate whether knowledge of integrin signalling involved in chondrogenesis could be used to improve cartilage regenerative medicine, looking at integrin expression during hMSCs differentiation in distinct culture model might broaden the understanding of the current impediments in cartilage tissue engineering.

The objective of this project was to understand MSCs adhesion requirements during chondrogenic differentiation. Furthermore, the information collected about integrin expression profile can be used to design more suitable material that would follow these adhesion requirements as reproducing adhesion ligand requirements could contribute to an optimized MSC differentiation in stable chondrocytes.

2.2. Native Cartilage Physiology

Cartilage is found in many places in the body, including the nose, ears, rib cage, and in between the bones in joints. Its role is to shape the body, and to protect bone ends by reducing wear in joints. Cartilage is a soft connective tissue composed of a highly specialized extracellular matrix and a single cell type, chondrocytes. There are three types of cartilaginous tissue which are distinguished by their extracellular matrix (ECM) composition, structure and mechanical properties (***Table 1***). The most

common type is hyaline cartilage, found in the larynx, trachea, bronchi, ribs, and on the surface of the bone in joints. Hyaline cartilage is mainly composed of collagen type II fibres. In the joint, it lines the bone surfaces providing a smooth and lubricating material to protect against joint wear, and absorbs the compressive forces exerted in the joint.[3] Fibrocartilage, another type of cartilage, is found in the meniscus and intervertebral discs for instance. It exhibits a high amount of collagen type I fibres oriented in order to resist high tensile strength. Compared to hyaline cartilage, it combines an important resistance to deformation and a greater resistance to traction forces due to the fibrous tissue. Finally, elastic cartilage displays an important flexibility owing to the presence of elastin fibres. Its role is to maintain shapes and resist to repetitive flexions. The outer ear, larynx and epiglottis are made of elastic cartilage.

Here, we are focusing on articular hyaline cartilage as the clinical need concerns mainly the regeneration of this type of cartilaginous tissue (Section 2.3). Whilst all types of cartilage have limited self-healing capacity, articular cartilage injury or degeneration leads to painful conditions and limits patients' movement. Indeed its dynamic and static functions are vital in the achievement of any movement: its dynamic role is to provide a friction-less surface between bones in joints in order to reduce wear, while its static function is to minimize and distribute stresses on the subchondral bone.[4] There is therefore a strong clinical demand to develop durable cartilage repair procedures as presently no appropriate solution is available.

Table 1 - Characteristics of the different cartilaginous tissues

Cartilage Type	Function	Characteristic Component
Hyaline cartilage	Reduce wear and resist to deformation	Primarily Collagen II
Fibrocartilage	Resist traction and deformation	Primarily Collagen I
Elastic cartilage	Highly deformable	Presence of Elastin

Chondrocytes are the only cell type present in cartilage. They are highly specialised, fully differentiated cells, sparsely distributed, and embedded in the ECM. Chondrocytes are trapped in lacunae surrounded by a 2 µm thick pericellular matrix (PCM) displaying a high concentration of collagen type VI normally absent in the regular cartilage ECM.[5] The cartilage cells together with the PCM form a structural and functional unit named chondron. Its precise role is not yet known, but is thought to play an important role in regulating biomechanical, biophysical, and biochemical interactions.[3,6]

Numerous factors influence chondrocyte physiology including stress,[7] hydrostatic pressure,[8,9] osmotic pressure, dynamic compression,[10,11] low oxygen tension,[12] and biochemical factors.[13,14] *In vivo* studies have demonstrated that mechanical loading significantly stimulates ECM production compared to a non-stimulated limb in the same animal. Although the mechanism of transduction is not yet well identified it would implicate integrins, but also stretch-activated ion channels. In addition to being recepients of signals from their environment, chondrocytes also actively remodel the cartilaginous tissue. They produce, maintain and remodel the tissue through anabolic and catabolic activities carried out by matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs).[15,16]

Overall, chondrocytes are very sensitive to their microenvironment, and can sense ECM composition or mechanical loading changes through several surface receptors, including integrins, and adapt their

activity accordingly. Consequently, it is particularly important to take into consideration their exquisite sensitivity and the need to cater to their environment when designing a material to support chondrocytes.

Cartilage is, in fact, mainly composed of extracellular matrix (ECM), with the chondrocytes representing only up 5% of the volume of the tissue.[17] The ECM of cartilage is extensively produced by the chondrocytes and is comprised of three main components: water, collagen type II fibres (except for the fibrocartilage which primarily contains collagen I) and proteoglycans (PGs), where aggrecan is the major PG found in hyaline cartilage. Over 100 chondroitin sulphate and keratin sulphate side chains, both glycosaminoglycans (GAGs), are covalently bound to aggrecan.[18] Furthermore aggrecan also interacts with hyaluronan to from huge proteoglycans aggregates. The resulting highly negative charge density endowed by the GAGs side chains together with their localisation in the ECM provides cartilage with the osmotic properties required to resist compressive loads (***Figure 1***).[19] Indeed, when cartilage deforms as it is subjected to an external load, water is expelled to areas under low pressure, leading to an increase of PG concentration in the high pressure areas and thus an increase of the osmotic pressure. The PGs repel each other due to their highly negative charge, thereby allowing absorption and distribution of mechanical stresses. When the load terminates, the high osmotic pressure draws the water back into that region, comparable to the behaviour of an elastic sponge.[4]

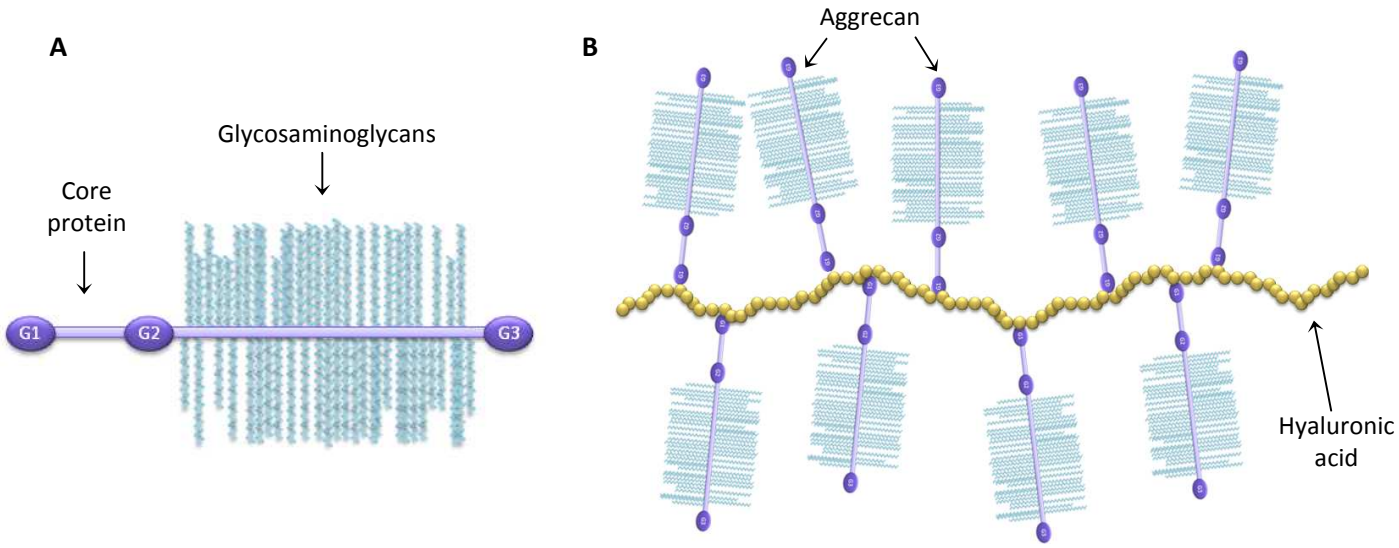


Figure 1 –Aggrecan (A) and proteoglycan aggregates (B). Glycosaminoglycan such as chondroitin sulfate and keratin sulfates are covalently bound to the core protein aggrecan, in turn aggrecan interacts with hyaluronic acid to form proteoglycans aggregates. During joint loading, waters is expelled from high pressure region in cartilage increasing PGs aggregates concentration hence increasing osmotic pressure. The water flows back to high osmotic pressure areas when loading terminates. Figure inspired by reference [18].

Articular hyaline cartilage ECM is composed of 68-85 wt% of water, 10-20 wt% of collagen type II and 5-10 wt% of aggrecan. Other components are present in lower quantities, including hyaluronan, link protein, collagen type I, V, VI, IX and XI, decorin, biglycan, fibromodulin, perlecan, thrombospondin and COMP.[5,20] It is an anisotropic and inhomogeneous tissue divided in three highly organised macroscopic regions: the superficial zone, the middle zone and the deep zone (***Figure 2***). Each zone is characterised by different structure, organisation and composition. The deep zone contains, for instance, more proteoglycans than the two overlying zones. Fibre orientation and chondrocyte

shape, size, gene expression, growth rate and activity vary depending on their location in the cartilaginous tissue.[21] Several studies tried to reproduce native cartilage complexity with engineering techniques, the outcomes seem promising, indeed bi-layered hydrogels showed enhanced mechanical properties than single-layered hydrogels when seeded with chondrocytes from superficial or deep zone alone.[22] However replicating native complexity is difficult, and we hypothesise that cell signalling through surface receptors is a major factor influencing chondrogenic differentiation and thus our project will focus on the role of surface receptors and their expression during chondrogenesis rather than attempting to mimic cartilage zonal organisation.

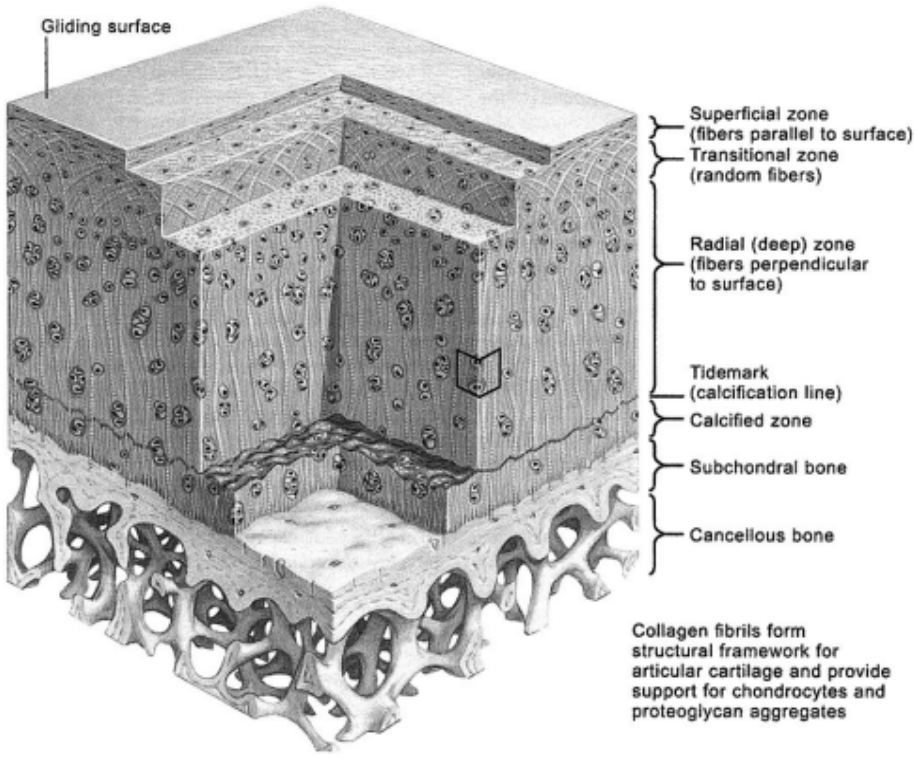


Figure 2 - Zonal organisation of articular cartilage. Hyaline cartilage is subdivided in three distinct zones characterized by structure and composition: the superficial zone, the middle zone and the deep zone. Collagen fibre alignment and chondrocyte phenotype depend on the localization in cartilage. Image from reference [4].

Cartilage does not contain any blood vessels. Chondrocytes are therefore maintained through nutrient diffusion rather than active nutrient transport, however the diffusion is helped by compression during joint loading. The mobility of the chondrocytes is very limited as they are located in lacunae, therefore chondrocytes are not able to migrate to the site of damaged cartilage to repair it. Because of its avascular nature, the limited supply of chondrocytes and their poor mobility, hyaline cartilage has low inherent capacity for auto-healing. It regenerates slowly and tends to produce fibrocartilage with lower mechanical properties than hyaline cartilage which leads to impaired function. This makes cartilage injury a major clinical issue and an exciting tissue engineering challenge. Small defects and defects penetrating the subchondral and can repair spontaneously with production of hyaline cartilage however for larger defects or for partial thickness defects fibrocartilage is produced.[23]

2.3. Cartilage Pathologies and Current Treatments

The most common degenerative cartilage disease is osteoarthritis (OA). OA is due to a disequilibrium of the anabolic and catabolic activities that occur in the articular hyaline cartilage.[24] The imbalance between degradation and synthesis of the cartilage leads to the loss of integrity of the tissue and thus to cartilage erosion which result in limited motion, pain or even disability. OA derives from an amalgam of numerous factors, including biochemical, biomechanical, inflammatory, and immunologic factors.[25] Over 14% of adults above 25 years are affected by OA however it is mostly patients above 45 years old who are affected by this condition (***Figure 3***).[26] Although OA is a major concern, as indeed,[25] cartilage damages involves also commonly traumatic sport accident, tear, trauma induces injuries, etc, and as aforementioned cartilage has a very low inherent healing capacity. Most of the time surgical procedures have to be considered to relieve the patient from pain and replace defective cartilage.

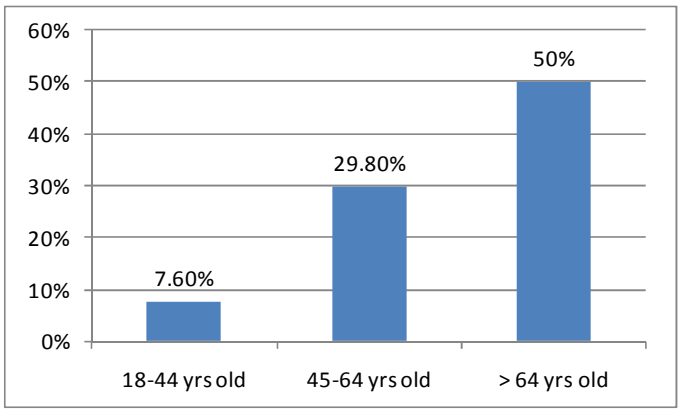


Figure 3 - Arthritis Prevalence in USA. One third of the adults between 45-64 years old experiences arthritis, over the age of 65 it affects half of the population.[26]

Currently, the most common treatments for small cartilage injuries are marrow stimulation techniques, osteochondral graft, and autologous chondrocytes implantation (ACI). For larger cartilage defects, joint replacement is considered. Although outcomes are promising, none of these procedures consistently result in clinically patent cartilage. In the marrow stimulation technique, the subchondral bone is penetrated at the bottom of the cartilage injury in order to fill the defect with blood and to induce bone-marrow derived stem cells to differentiate into chondrocytes in order to synthesise cartilage. However these cells are rare and their availability is patient age-dependant. Importantly, fibrous tissue is produced rather than hyaline cartilage and due to its inferior biomechanical properties a rapid degeneration occurs in the repaired tissue and deterioration of the clinical results begins after 18 months.[27,28] The osteochondral graft is a procedure where a cartilage plug with its subchondral bone is transplanted to the damaged site from a non loading bearing site or from a cadaver. Although hyaline cartilage is obtained through this method, it involves donor site morbidity and is only suitable for small defects.[23] Another well-known and established treatment is autologous chondrocytes implantation, where chondrocytes are extracted from patient's cartilage, expanded *in vitro* for several weeks and eventually injected into the damaged site. However chondrocytes tend to dedifferentiate *in vitro* which leads to synthesis of fibrous cartilage. Moreover, the size of the defect that can be treated with ACI is limited and cannot be applied to large defects.[29,30] Finally, for major injuries, joint replacement is considered but while the outcomes are encouraging, the prostheses eventually fail overtime especially in young and active patients. Overall,

due to the production of fibrocartilage, risk of infection, donor site morbidity and major surgical procedure along with long term rehabilitation, these solutions are far from being optimal. Furthermore, the need for treatment for cartilage repair is constantly increasing due to the ageing and more active population, along with the growing obesity issue which leads to an increase in degenerative joint disease. Indeed over 250,000 knee and hip replacement are performed per year in US and this number does not include the small cartilage injuries.[17]

From this brief review of currently used techniques, it becomes clear that there is a strong clinical need for cartilage tissue engineering which would allow treatment of small acute defects but also larger defects.

2.4. Cartilage Tissue Engineering Approach and the Use of Mesenchymal Stem Cells

There is a clear need to develop an innovative approach from the state of the art of the current treatments available for cartilage injuries. Recently, regenerative medicine has been considered a viable option to meet the clinical need for cartilage. Regenerative medicine or tissue engineering is a multidisciplinary field that applies engineering principles to life science in order to replace or repair a tissue. More specifically, cartilage tissue engineering intends to reproduce hyaline cartilage with similar properties, structure and organisation to the native cartilage through an appropriate combination of cell type, preferably autologous cells, biomaterials and signalling factors.[17] Because of the low inherent healing capacity of cartilaginous tissue, considerable efforts in the area of cartilage tissue engineering and have yielded a number of new materials and cellular approaches. Stem cells, and more specifically mesenchymal stem cells (MSCs), are a particularly promising cell candidate for cartilage defect repair due to their ability to circumvent the limited supply of autologous chondrocytes. MSCs have been shown to successfully undergo chondrogenesis *in vitro* and Ashton *et al.* were the first to report *in vivo* chondrogenesis. Important work that followed demonstrated the potential of inducing chondrogenesis of MSCs with defined chondrogenic medium containing dexamethasone and transforming growth factor-beta (TGF-β).[23,31,32] Since then, many different protocols have been elaborated to direct MSCs differentiation toward chondrocytes both *in vivo* and *in vitro*, particularly a combination of TGF-β with bone morphogenetic proteins (BMPs) provided excellent results.[33]

MSCs are suitable candidates for cartilage engineering as they are adult multipotent stem cells with a high self-renewal capacity which allows a significant *in vitro* expansion while retaining their stem cell-like characteristics. Furthermore, MSCs were shown to differentiate into various cell types under appropriate culture conditions including osteocytes, chondrocytes, adipocytes, tenocytes, myocytes, neurons, hepatocytes, cardiomycoytes, and astrocytes.[23,34-39] These cells are rather rare; in bone marrow extract it is estimated that only 1 out of 10000 nucleated cells is a MSC. However, they can be isolated from numerous tissues among which bone marrow, trabecular bone, skeletal muscle, fat tissue, periosteum, and synovium,[40-43] which makes them easily available. Indeed, the frequency of appearance in these tissues is over 100-fold higher than in bone marrow extract.[44] Although MSCs extracted from different tissues show similar behaviour, they have specific properties which are related to their tissue origin. Huang *et al.* studied the chondrogenic potential of progenitor cells derived from bone marrow and adipose tissue, and demonstrated that the tissue synthesised by

bone marrow-derived cells was more cartilaginous in comparison to the tissue produced by adipose tissue-derived cells as shown by higher accumulation of collagen type II and GAGs.[45] Another study conducted by Sakaguchi *et al.* compared MSCs derived from five different mesenchymal tissues: bone marrow, synovium, periosteum, skeletal muscle, and adipose tissue. Significant differences were found among the MSCs when looking at in their proliferation and differentiation abilities, in similar conditions synovium-derived cells were superior to other tissue-derived cells in terms of multipotentiality.[44] However for chondrogenesis purposes, bone marrow-derived MSC produced an equal amount of ECM than synovium-derived cells, making them an appropriate choice for our study.

The isolation process of MSCs is challenging as the phenotypic identity is neither unique nor fully characterised. These cells share features of multiple cell lineages including endothelial, epithelial, hematopoietic, and muscle cells. Yet, no specific surface markers have been identified, thus isolation is mostly done through negative selection.[46] STRO-1 was initially reported as a surface marker for bone marrow-derived MSCs,[47] however other studies showed bone marrow-derived MSCs were negative STRO-1,[48] which illustrates how imprecise and contradictory the information about MSC surface markers can render their isolation particularly challenging. Yet, the easy availability of MSCs together with their high proliferative potential and multipotency make them appropriate candidates for cartilage tissue engineering in order to substitute chondrocytes. Additionally, the use of autologous MSC avoids the graft rejection issue. In our study, we used commercially available human MSCs.

2.5. Current Limitations of Cartilage Tissue Engineering

Although some noticeable advances have been made in cartilage tissue engineering, the properties and structure of native cartilage have not been completely reproduced. MSCs tend to undergo hypertrophy as a terminal differentiation rather than becoming stable chondrocytes. To date, even if chondrogenesis from MSCs has been achieved *in vitro*, many parameters need to be improved in order to achieve functioning tissue engineered cartilage beyond simply obtaining chondrocytes.

For example, R.L. Mauck *et al.* did a comparative study of the efficiency of matrix formation between bovine MSC-derived chondrocytes and healthy mature chondrocytes from the same donor in long-term agarose culture.[49] Results showed that the amounts along with the mechanical integrity and properties of the matrix produced by MSCs having undergone chondrogenesis were lower than mature chondrocytes matrix under the same conditions. This suggests there is an inherent reduction in efficiency due to *in vitro* chondrogenesis which is independent from the biomaterial used. New protocols need to circumvent this drawback before using the differentiated cells in a clinical level. Undeniably, cartilage tissue engineering is facing some important issues. MSC-derived chondrocytes have different phenotypes than native chondrocytes, and despite using numerous growth factors, they are hypertrophic and express fibrocartilage markers. *In vivo,* chondrocytes are known to express proteins such as collagen II, SOX9, aggrecan, COMP, decorin and biglycan.[14] However, *in vitro* MSC chondrogenesis leads to expression of fibrocartilage-features, including collagen I[50], and hypertrophic markers, collagen X, alkaline phosphatase (ALP) and matrix metalloproteinase 13 (MMP13) [14,51]. Ichinose *et al*. investigated collagen I, II, X and proteoglycan expression in hMSCs entrapped in alginate beads with chondrogenic medium for a period of 19 days.[52] Collagen II, X and proteoglycan expression increased the first eight days, but from days 8 through 19, collagen II and proteoglycan

expression dropped while collagen X expression was maintained. They concluded that hMSCs rapidly differentiate into chondrocytes which ultimately undergo hypertrophy. Peltari *et al.* found that in pellet culture, both hypertrophic markers collagen X and MMP13 were upregulated before collagen II.[51] All together, it is clear that *in vitro* chondrogenesis of MSCs it not comparable to developmental processes *in vivo*.

Until now, MSC-derived chondrocytes obtained through common *in vitro* chondrogenesis protocols showed a different phenotype than mature chondrocytes. They tend to produce ECM proteins related to fibrocartilage instead of hyaline cartilage, as shown by high expression of collagen type I, and eventually undergo hypertrophy. It seems that the protocols today available produce a cartilage similar to the transient endochondral cartilage rather than a stable hyaline cartilaginous tissue, although no mineralization was detected *in vitro,*[27] probably because *in vitro* the TGF-β repress the terminal differentiation as it retains the chondrocytes in the pre-hypertrophic state.[53] Undoubtedly MSC chondrogenesis *in vitro* needs to be optimized to get a matrix with similar properties to its native hyaline cartilage counterpart. The great cartilage tissue engineering challenge is to obtain stable chondrocytes that do not undergo hypertrophy and produce hyaline cartilage ECM molecules.

2.6. Importance of the Extracellular Matrix

2.6.1. Influence of the Extracellular Matrix on Cell Behaviour

The role of the extracellular matrix is not only to provide a mechanical support to the tissue and the cells but to regulate fundamental cell function. It has been shown that cell-matrix interactions are essential for cell survival and that they modulate cell behaviour. By determining cell shape ECM can regulates numerous cellular processes. Interactions with ECM determine the intracellular organisation of the cytoskeleton which in turn determines the cell shape thus controls indirectly cell growth, proliferation, differentiation, gene expression and secretion but also cell survival. Indeed a couple of decades ago, Meredith *et al*. investigated the role of extracellular matrix as a cell survival factor, their study revealed that cells tend to rapidly undergo apoptosis in the absence of any cell-ECM interactions and that integrins are involved in the process.[54] Particularly, chondrogenesis is highly dependent on cell shape, indeed the round morphology is required to initiate the differentiation *in vivo*.[55]

To summarise, cell-matrix interactions modulate numerous vital cell processes by regulating cytoskeleton organisation and by activating different intracellular signalling pathways. Moreover the major surface receptor family mediating these interactions is the integrin family and thus they are of great importance in regulating cell behaviour.

2.6.2. The Integrin Family

Integrins are cell adhesion receptors involved in both cell-matrix and cell-cell interactions. They are αβ heterodimeric transmembrane glycoproteins which relate the extracellular matrix to the intracellular machinery. Integrins are not only important for the integrity of the tissue since they are physical linkages between the ECM and the cytoskeleton, but importantly, they also trigger intracellular signals which regulate cell behaviour and physiology.

Table 2 - Vertebrate Integrin Subunits

List of integrin subunits	
α subunits	α1, α2, α3, α4, α5, α6, α7, α8, α9, α10, α11, αD, αE, αL, αM, αV, αW, αX
β subunits	β1, β2, β3, β4, β5, β6, β7, β8

To date, 18 alpha subunits and 8 beta subunits were identified in mammals and these assemble in a non-covalent manner to form 24 different integrins with specific ligand properties (***Table 2***).[56] The heterodimerisation of the two subunits occurs intracellularly thus no free α or β subunits are found on the surface of the cell. The specificity of the integrin to a ligand is determined by the α subunit which is composed of 7-bladed β-propeller, a thigh, a calf-1, and a calf-2 domain, half of the 18 α subunits have an I domain inserted between blade 2 and 3 in the β propeller region which binds the ligand. The β subunit is composed of a plexin-sempahorin-integrin domain (PSI), a hybrid domain, a βI domain and four epidermal growth factor repeats (EGF) (***Figure 4***). If the α subunit does not contain an I domain, the ligand binding occurs through the βI domain which complex with the 7-bladded β propeller of the α subunit, moreover the βI domain contains a region binding divalent cations involved in the activation of the integrin.[57]

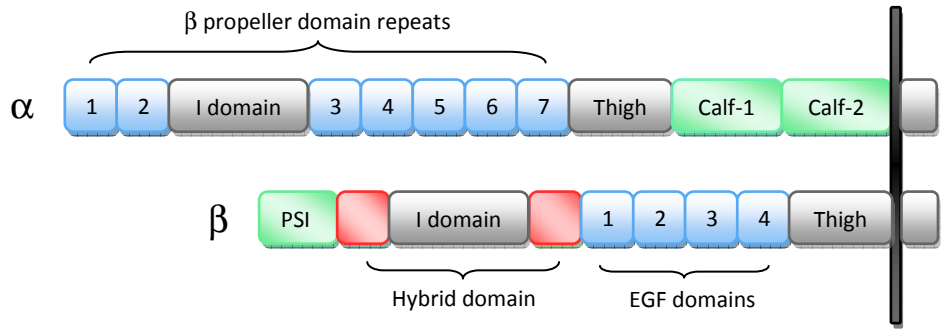


Figure 4 – Representation of an αI domain containing integrin. Only nine out of the 18 different α subunits contains an αI, all integrins contain a βI domain in their beta subunit. Figure adapted from reference [56].

The integrin family can be classified in different subgroups depending on the ligand they bind or on their subunit composition, ***Figure 5*** summarises the complete mammalian set and their major ligands specificities, however this figure is highly simplified as it is well known that many integrins are promiscuous and bind more than one ligand. In green are depicted the integrins recognizing the RGD tripeptide sequence found in fibronectin and vitronectin, in purple are the integrins binding laminin, in blue integrins binding collagen are represented, finally in pink are the integrins recognizing Ig-superfamily receptor and which are thus involved in cell-cell interactions, α4β1 and α9β1 recognise both fibronectin and Ig-superfamily receptors.[2]

The extracellular domain of the integrin binds the ligands, usually ECM proteins but may binds with soluble cues or other cell surface receptors, and determines the specificity of the integrin. Both α and β subunits are involved in ligand recognition, however the alpha subunit plays a central role in integrin specificity. The intracellular domain interacts with the cytoskeleton via the beta subunit and a variety of intracellular proteins among which include talin, kindlin, and vinculin. The intracellular domain is very short, typically less than 50 amino acids, but plays a key functional as it links ECM to

the cytoskeleton through numerous intermediate proteins in order to trigger intracellular signals, all known β subunits bind to actin filaments with an exception for α6β4 which binds to intermediate filaments.[58,59]

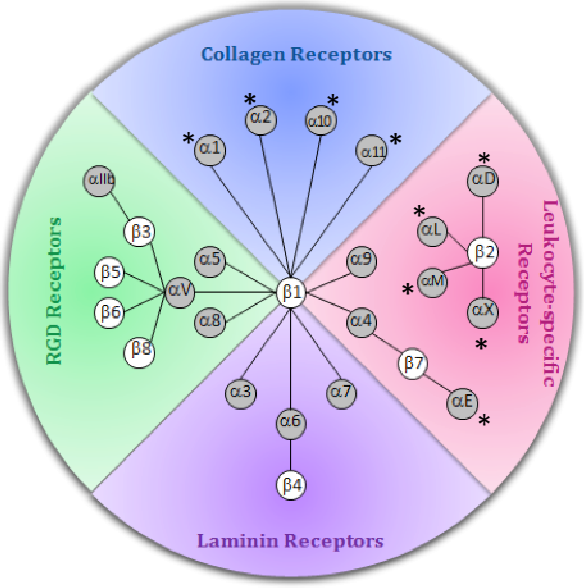


Figure 5 - The Integrin Family. 18 α subunit and 8 β subunits assemble to form 24 distinct heterodimeric integrins with different ligand properties. Integrins can be classified in subfamilies based on ligand specificity (four subgroups are represented) or evolutionary relationships. β2 and β7 expression is mostly restricted to white blood cells. α4β1 and α9β1 recognise both fibronectin and Ig-superfamily receptors. The α subunits containing an αI are represented with a star (∗). Figure redrawn from reference [2].

It is important to remember that integrins are not only important transmembrane mechanical links for cellular adhesion but also involved in activating intracellular signalling pathways. It is well known that cell-matrix interactions are required for cell survival as they modulate cell behaviour[54], their binding triggers intracellular pathways which modulate numerous vital cell processes including proliferation, shape, motility, polarity, survival, apoptosis, gene expression, matrix remodelling, and differentiation.[2,60] Integrin-ligand binding triggers transduction events very similar to those triggered by growth factors. Moreover, integrin signalling pathways involve many different transcription factors which influence key genes expression and affect cellular mechanisms. Complex signalling launched by integrins involves numerous intracellular proteins modulating cell behaviour. ***Figure 6*** summarises the main pathways and regulation of cells processes.

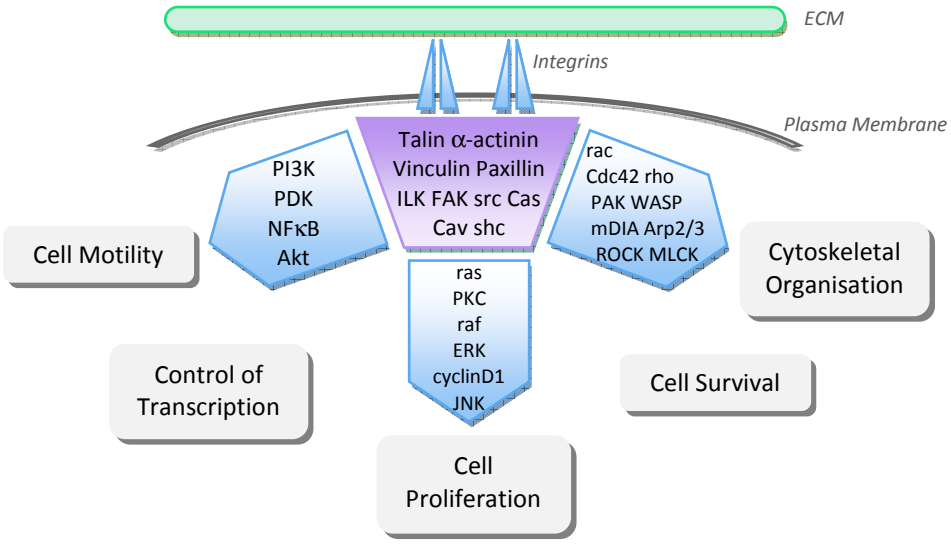


Figure 6 - Integrin Signalling. Integrins influence numerous vital cell processes such as cell shape, migration, survival, differentiation, gene expression, etc. The major pathways by which signal transduction occurs are not completely elucidated, some of the main pathways are summarised in this figure. Figure adapted from Hynes' work[2].

An important aspect of the integrin family is their bidirectional signalling ability: the outside-in and the inside-out signalling.[2] Extracellular ligand binding to integrins induces conformational changes and clustering of the adhesion receptors which in turn generate intracellular signals which regulate major cell processes and is named the outside-in signalling (***Figure 6*** and ***Figure 8***). Conversely, the inside-out signalling is the activation of integrin upon information coming from within the cells. Indeed it is known that most of the integrins, if not all, are found on the cell surface in an inactive state and needs to be activated in order to bind any ligand, through separation of alpha and beta cytoplasmic tails. The intracellular regulation of integrin function is known as the inside-out signalling (***Figure 7***).

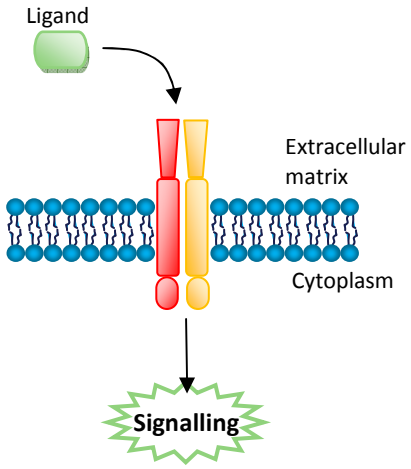


Figure 8 - Outside-in Signalling. Integrins are not only adhesion receptors, they also transmit information from en extracellular environment to the cell by triggering signaling pathways upon binding to its ligand. Figure inspired by reference [1].

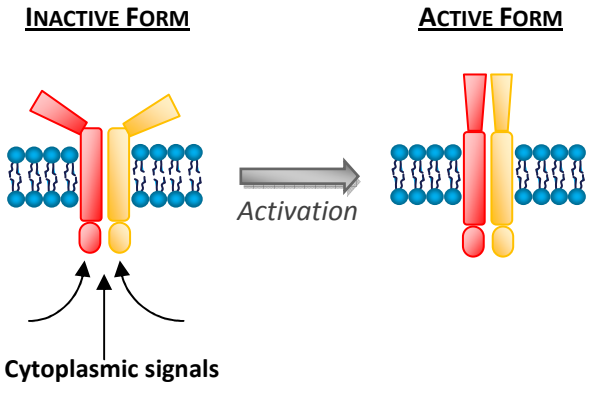


Figure 7 - Inside-out Signalling. Integrins can be activated from within the cells in response to intracellular events, this process is known as inside-out signalling and regulates integrin affinity for adhesive ligands. Figure inspired by reference [1].

In conclusion, integrins should be seen both as mechanical linkages between cell and ECM and as bidirectional signalling receptors, this dual role makes them essential in regulating cell behaviour and are fundamental to numerous vital cellular processes. They are also often referred as mechanotransducers as they are involved in the transduction of mechanical stimulus from the ECM in biochemical intracellular signals.

2.6.3. State of the Art of Cell-Matrix Interactions Mediated by Integrins during Chondrogenesis

In this project, we are interested into cell-matrix interactions mediated by integrins and their implication in MSC chondrogenic differentiation. As aforementioned, ECM interactions are necessary to initiate chondrogenesis. Indeed these interactions are primordial, even more essential than cell-cell interactions for chondrogenic differentiation. Solursh *et al*. cultured isolated mesenchyme limb cells which had undergone chondrogenesis and expressed chondrocyte phenotype markers under appropriate conditions. The differentiation occurred only when cells where cultured in a round configuration, it appeared that cell shape and thus indirectly cell-ECM interactions are of major importance for chondrogenic differentiation.[61] In their natural environment, single chondrocytes are trapped in lacunae and only have small processes that extent into the matrix but do not interact with neighbouring cells.[20] Undeniably, chondrocytes interact strongly with their direct micro-environment and this is why three dimensional (3D) culturing systems are particularly appreciated for *in vitro* chondrogenesis as it mimics more closely *in vivo* conditions and maintains the round morphology of the cells as culture conditions flattening cell morphology are known to encourage chondrocyte dedifferentiation. Additionally, it is well established that chondrocytes cultured in monolayer at low density rapidly dedifferentiate, conversely it was shown that 3D culture systems stabilize the chondrocytic phenotype.[62]

As aforementioned, cell-ECM interactions modulate fundamental cell functions including differentiation. As integrins mediate these interactions they have been extensively studied during MSC differentiation. Their role in chondrogenesis was also greatly investigated although it not clear yet how they influence the differentiation. Varas *et al*. studied the expression of $\alpha 10$ and $\alpha 11$ subunits during hMSC chondrogenesis and discovered that $\alpha 10$ was upregulated while $\alpha 11$ and $\alpha 1$ were downregulated over time.[63] They found that cells highly expressing $\alpha 10$ subunit had a high chondrogenic differentiation potential, and proposed $\alpha 10$ as a potential marker to predict MSCs differentiation state, with $\alpha 11$ being a mesenchymal tissue marker. The expression of fibronectin, laminin and their related integrins $\alpha 3\beta 1$, $\alpha 5\beta 1$ and $\alpha 6\beta 1$ during MSC chondrogenic differentiation were also investigated by Tavella *et al.*[64]. Their study showed a clear temporal hierarchy in the regulation of fibronectin, laminin and their corresponding integrins. It has been proven that integrins have an essential role in the regulation of chondrogenesis. In fact, Hirsch *et al.* blocked integrin signalling using antibodies against $\alpha 2$, $\alpha 3$ or $\beta 1$ integrin subunits which was shown to suppress chondrogenic differentiation and lower chondrocyte survival.[65] *In vivo,* $\beta 1$ tissue-specific knock-out causes a severe cartilage phenotype, reduced adhesion to extracellular components, disorganised growth plate due to a diminished motility and defective cell division cycle leading to reduced proliferation.[66] Furthermore, *in vivo* knocking out the $\alpha 10$ subunit led to defects in the growth plate along with abnormal chondrocyte morphology and reduced proliferation[67], while $\alpha 1$ subunit knock-out led to osteoarthritis.[68]

These results revealed the importance of interactions mediated by integrins between chondrocytes and their surrounding extracellular matrix, which also influences integrin expression throughout MSC chondrogenesis.

2.7. Investigating Integrin Expression During MSCs Chondrogenesis

Cartilage tissue engineering research has been mostly focused on mimicking the physical properties of native cartilage extracellular environment. Architecture can be reproduced at micrometer and nanometer scale, scaffold stiffness and oxygen tension can be matched to native cartilage matrix. However, with the exception of decellularised scaffolds, our strategies are poor at mimicking the biological properties. This study aims to provide information that can be used to better match at least one aspect of the biological complexity present in the native extracellular matrix.

In the present work, we investigated the changing adhesion requirements of bone marrow-derived MSCs during chondrogenesis by looking at integrin transcript expression over the time course of chondrogenesis. Our aim was to identify a temporal transcript expression for each integrin subunit during the chondrogenic differentiation.

As aforementioned, integrins are of major importance throughout chondrogenesis, they modulate the differentiation and stabilise the chondrocyte phenotype. Our hypothesis was that integrin expression varies during chondrogenesis, as several studies have shown,[63] which therefore the adhesion ligand requirements of MSCs change as they become chondrocytes. Although other studies have also focused on integrin expression during chondrogenic differentiation, most of them only analysed one or two integrins simultaneously and looked at only one culture system, or looked at several integrins but only at the end point of chondrogenesis. In this project, we are interested at following the integrin transcript expression in different cell culture methods or scaffolds, and we have investigated thus integrin expression profile in three different systems. The objective of this project was to understand MSCs adhesion requirements during chondrogenic differentiation and to contribute to cartilage tissue engineering by informing the future design of more suitable material that would cater to these adhesion requirements.

2.7.1. Culture Systems and Cell Source

The first step was the selection the cell type to induce chondrogenic differentiation along with the different culture models. As previously mentioned hMSCs are promising candidates for chondrogenesis as they can be easily isolated from the patient, and are capable of both self-renewal and chondrogenic differentiation. hMSCs are available from numerous tissue and they have specific properties which are related to their tissue origin, for chondrogenesis synovium and bone marrow derived hMSCs are the most appropriate cells. We opted for bone marrow-derived hMSC as it is the best characterised population.[23,44]

We decided to investigate integrin transcript expression in three distinct 3D high-density cell systems. 3D systems are known to encourage chondrogenesis and stabilise chondrocyte phenotype by mimicking more closely in vivo conditions and promoting cell-matrix interactions, while 2D cultures lead to chondrocytes dedifferentiation.[69] The 3D systems investigated are:

- pellet culture,

- micromass culture, and
- collagen type II gels,

Pellet culture, prepared by centrifugation is currently the gold standard method for hMSCs differentiation into chondrocytes, yet differentiation was shown to be heterogeneous and nutrient diffusion in the core of the pellet is hindered by matrix and a very high cell density leading to a necrotic core. Hence we decided to look at integrin transcript expression in micromass culture which is similar to pellet culture however micromass are formed by high density cell suspension the cell density is thus lower than in pellet which allow a better nutrient diffusion to the core of the micromass. Moreover micromass culture was shown to be more efficient for hMSC chondrogenesis.[69] Finally, the last culture system investigated is collagen type II 3D hydrogels, Bosnakovski *et al*. investigate hMSC chondrogenic differentiation in collagen I, collagen II and alginate gels and showed that collagen type II gels showed the best results.[70] Collagen II, the major component found in native hyaline cartilage supports and promotes chondrogenesis of MSCs due to its inherent biological cues for chondrocytes. Furthermore, cells are easily harvested in mild conditions from collagen type II heat gels from, making it an appropriate material for gene expression profiling as it is known that gene expression is rapidly modified under cellular stress.

2.7.2. Screening Integrin Subunit Expression at Different Time Points

Integrin transcript expression was evaluated using quantitative real-time polymerase chain reaction (qRT-PCR). All known integrins subunits expression was studied by qRT-PCT: 18 α subunits and 8 β subunits. Moreover the expression of chondrocyte phenotype markers was assessed: *ACAN, SOX9* and *COL1A1, COL2A1* and *COL10A1*. Other cell lineage markers were studied: *PPARG* for adipogenesis and *RUNX2* for osteogenesis.

MSCs were cultured in either growth medium or chondrogenic medium for a period of 21 days and we chose 6 time points over this period to follow the variations in integrin transcript expression, hence expression was evaluated on Day 0, 1, 2, 4, 7, 14 and 21.

3. Materials and Methods

3.1. Routine Cell Culture

Cryopreserved Passage 1 human mesenchymal stem cells derived from bone marrow were purchased from PromoCell (UK). Cells were seeded at 4000 cells/cm^2 in tissue culture flasks and were expanded for two passages in hMSC-optimised growth media obtained from PromoCell, which was changed every two days. Approximately 25,000 cells/cm^2 were typically observed at confluency. Cells were frozen at Passage 3 and after thawing, were passaged once before being used in the experiments.

- *Freezing cells*: Cells were harvested from tissue culture flasks and resuspended in freezing medium from Promocell (UK) at 10^6 cells/ml. 1 ml was transferred to cryovials and stored in a freezing container containing isopropyl alcohol at -80°C overnight (freezing rate of approximately -1°C/min). Vials were then transferred to the vapour phase of liquid nitrogen for long-term storage.
- *Thawing cells:* Cryovials were removed from the liquid nitrogen container and submerged into a water bath at 37°C for 90 sec. Cells were then transferred to a 50 ml polypropylene conical tube, where pre-warmed hMSC growth medium was gradually added. Cells were seeded in culture flasks at a density of 4000 cells/cm^2.

3.1.1. Pellet Culture

hMSC pellet culture was carried out following a protocol by Reger *et. al*. Passage 4 hMSCs were trypsinised and aliquots of 2.5 x 10^5 cells were suspended in 500 µl of hMSC chondrogenic medium or hMSC growth medium (Promocell, UK). The pellets were formed by centrifugation at 450 x g for 10 minutes in 15 ml polypropylene conical tubes. Cells were cultured at 37°C and 5% CO_2 with loosened caps for gas exchange and medium was changed every three days. Cells were harvested on Day 0, 1, 2, 4, 7, 14 and 21 for quantitative polymerase chain reaction (qPCR) or histology.

3.1.2. Micromass Culture

Micromass culture was performed following the protocol recommended by Promocell [*Chondrogenic Differentiation and Analysis of MSC, Application note*]. Passage 4 hMSCs were harvested and resuspended in hMSC growth medium at a concentration of 2.5 x 10^5 cells per 200 µl in each well of a 96-well U-bottom suspension culture plate. After 48 hours, spheroids spontaneously formed and chondrogenesis was induced using hMSC chondrogenic medium (Promocell, UK). This was designated as Day 0. Medium was changed every second or third day. Cells were harvested on Day 0, 1, 2, 4, 7, 14 and 21 for quantitative polymerase chain reaction (qPCR) or histology.

3.1.3. 3D Collagen II Gels

Collagen II heat gels of a final volume 125 µl were prepared in a 96-well plate with 2.5 x 10^5 Passage 4 hMSCs suspended within the gel. Acidic bovine articular cartilage-derived collagen II (BD Biosciences, UK) was diluted on ice with chondrogenic medium or hMSC growth medium (Promocell, UK) to a final concentration of 1.5 mg/ml and 25 µl of HEPES buffer was added per 1 ml. The mixture was then neutralised with NaOH to reach a pH of 7.4 and gelation occurred at 37°C. Hydrogels were incubated at 37°C and 5% CO_2 for 4 to 5 hours then 0.125 µl of hMSC growth or chondrogenic medium was added to each well. Medium was changed every second day. Cells were harvested on Day 0, 1, 2, 4, 7, 14 and 21 for quantitative polymerase chain reaction (qPCR) or histology.

3.2. RNA Extraction

3.2.1. Pellet culture, Micromass Culture and Collagen II 3D Gels

Total RNA was isolated from pellets using QIAGEN RNeasy Mini kit (QIAGEN, UK) following the manufacturer's protocol. Pellets, micromass or collagen gels were washed two times in phosphate buffered saline (PBS) and 350 µl of RLT lysis buffer (with 1% (v/v) of β-mercaptoethanol) was added. The sample was lysed by pipetting or vortexing and stored at -20°C prior RNA extraction. The quality and purity of isolated RNA was assessed by spectrophotometry using the Nanodrop 2000c (Thermo Scientific, UK). In this technique, the UV absorbance of the samples is measured at critical wavelengths: 280 nm for DNA absorbance, 260 nm for RNA absorbance and 230nm for background absorbance including salt and proteins. Optical density (OD) is used to quantify RNA and given a known extinction coefficient, gives a concentration in ng/µl. The ratio $\frac{A260}{A280} = \frac{RNA\ absorbance}{DNA\ absorbance}$ assesses RNA purity and its value should be ideally situated between 1.8 and 2. Another ratio should be considered: $\frac{A260}{A230} = \frac{RNA\ absorbance}{Background\ absorbance}$, its value should also be situated between 1.8 and 2, a lower value indicates contaminants such as proteins or salts. The $\frac{A260}{A230}$ ratio is critical when extracting small amount of RNA, as the value can easily drop under 1.

3.3. Molecular Biology Tools for Integrin Expression

3.3.1. cDNA Synthesis

First-strand cDNA synthesis and quantitative polymerase chain reaction were performed using Superscript® III Platinium® from Invitrogen (UK). Approximately 100 ng total RNA was reverse transcribed in a 20 µl reaction volume. The thermocycling programme was 25° for 10 min, 50°C for 30 min and 85°C for 5 min. Following first-strand synthesis, the sample was treated with RNase.

3.3.2. Quantitative Polymerase Chain Reaction (PCR)

Quantitative PCR was performed in a 10 µl final volume with 200 nM of each primer and 2.5 µl diluted (1:100) cDNA. Thermocycling was carried out for 40 cycles at 95°C for 15 s and 62°C for 30 s in a Rotor-Gene 6000 (Corbett Life Science, UK). For amplification of ITGA4 and ITGA6, an annealing/elongation temperature of 63°C was required. Each reaction was performed in technical triplicates.

2-steps cycling qPCR	
Hold	50°C for 2 min
Hold	95°C for 2 min
40 cycles	➢ 95°C, 15s ➢ 62°C or 63°C, 30s
Melt curve	Temperature raised stepwise from 70 to 95°C, 1°C per 5 s step.

3.3.3. qPCR Data Analysis: ΔC_T and $\Delta\Delta C_T$ Methods

There are two main methods to analyse data obtained from real time qPCR: the absolute quantification which determines an input copy number and the relative quantification where the change in gene expression is compared to a reference. In this study, the reference was Passage 4

hMSCs cultured on tissue culture plastic (i.e. - undifferentiated hMSCs). Datas were analysed with the $\Delta\Delta C_T$ method for integrin subunit expression and with the ΔC_T method for phenotype markers as most of the latter were simply not expressed in the undifferentiated hMSC.

The $\Delta\Delta C_T$ method

As stated in Livak *et al*.'s paper,[71] the exponential amplification of the cDNA in PCR is described by the following expression:

$$A_n = A_0 \cdot (1 + E_A)^n \qquad eq.1$$

Where A_n the number of target cDNA copies at the cycle n of the reaction, A_0 is the initial number of copies, E_A the efficiency of the amplification defined during primer validation. At the threshold cycle C_T the fluorescence reaches a fixed threshold which is determined by the number of cDNA copies or A_T:

$$A_T = A_0 \cdot (1 + E_A)^{C_T} \qquad eq.2$$

Similarly for the reference gene (the internal control) that will be use to normalise the PCRs as difference in C_T might be due to differences in initial cDNA amount, we have:

$$R_n = R_0 \cdot (1 + E_R)^n \qquad eq.3$$

At the threshold cycle C_T:

$$R_T = R_0 \cdot (1 + E_R)^{C_T} \qquad eq.4$$

Now we assume that the efficiency of the target and the reference are the same E_R=E_A=E, dividing A_T by R_T gives:

$$\frac{A_T}{R_T} = \frac{A_0 \cdot (1 + E_A)^{C_T}}{R_0 \cdot (1 + E_R)^{C_T}} = A_{norm.} \cdot (1 + E)^{C_{T,A} - C_{T,R}} = A_{norm.} \cdot (1 + E)^{\Delta C_T} = K \qquad eq.5$$

Where C_T is the difference between the C_T of target A and the C_T of the reference R:

$$\Delta C_T = C_{T,A} - C_{T,R}$$

Where K is a constant, A_{norm} is equal to A_0/R_0 the normalized amount of target, and ΔC_T the difference in threshold cycles between the target and the reference. If we simply rearranged the equation we have:

$$A_{norm.} = K \cdot (1 + E)^{-\Delta C_T} \qquad eq.6$$

Finally, the normalized amount A_{norm} of a sample X is compared to the normalized amount of a sample a calibrator $A_{norm,\,cb.}$:

$$\frac{A_{norm.\ X}}{A_{norm.\ cb}} = \frac{K \cdot (1 + E)^{-\Delta C_{T,X}}}{K \cdot (1 + E)^{-\Delta C_{T,cb}}} = (1 + E)^{-\Delta\Delta C_T} \qquad eq.7$$

Where $\Delta\Delta C_T$ is :

$$-\Delta\Delta C_T = -\left(\Delta C_{T,X} - C_{T,cb}\right)$$

Finally if the efficiency is close to one, E = 1, the equation simply becomes:

$$\frac{A_{norm.\ X}}{A_{norm.\ cb}} = (1+E)^{-\Delta\Delta C_T} = 2^{-\Delta\Delta C_T} = amount\ of\ target \qquad eq.8$$

The final result is thus normalised to a reference, or internal control, and expresses a time fold difference in expression between the sample X and the calibrator sample.

The reference or internal control is usually a housekeeping gene and the calibrator is an untreated control. In our study glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen as a housekeeping gene and the calibrator sample is Passage 4 hMSCs before trypsinisation from tissue culture plastic (TCP). Hence the data are presented as a time fold change relative to the target gene expression in the calibrator sample, and the value obtained can vary from 0 to infinity:

$0 \leq data < 1$	$data = 1$	$1 < data < \infty$
Less expressed than in hMSCs cultured on tissue culture plastic (TCP)	*Same expression as in hMSCs cultured on TCP*	*More expressed than in hMSCs cultured on TCP*

Assumptions of $\Delta\Delta C_T$ methods:

The $\Delta\Delta C_T$ method assumes that the amplification efficiency of the target and reference genes are similar and close to 1, however this is an ideal situation. The efficiency is usually not equal due to difference in PCR product size, difference in primer annealing efficiency and GC content. The error is a function of the PCR efficiency, E, and cycle number, n, and is calculated in % with the following equation:

$$\%\ Error = \left(\frac{2}{1+E}\right)^{n} \cdot 100 - 100 \qquad eq.9$$

The $\Delta\Delta C_T$ method should only be chosen to analyse data if the efficiency between the target gene and reference gene are similar, a difference of less than 10% between the two efficiencies is considered acceptable.[72] Obviously small differences of expression will fall in the 10% error and are not seen.

The ΔC_T method

The $\Delta\Delta C_T$ method does not apply to genes not expressed in hMSCs cultured on TCP, as the transcript expression cannot be expressed relative to the normalising sample. For the phenotypes markers, data are expressed as fold-change relative to the housekeeping gene (GAPDH) in the same sample using the ΔC_T method instead of the $\Delta\Delta C_T$. The data are simply not compared to a calibrator simple thus equation 6 from the $\Delta\Delta C_T$ above applies:

$$A_{norm.} = K \cdot (1+E)^{-\Delta C_T} \qquad eq.6$$

Where:

$$\Delta C_T = C_{T,gene\ of\ interest\ A} - C_{T,reference\ gene\ R}$$

Assuming an efficiency of 1:

$$A_{norm.} = K \cdot 2^{-\Delta C_T} \qquad eq.10$$

The data are expressed as a time fold between 0 and infinity relative to GAPDH expression:

$0 \leq data < 1$	$data = 1$	$1 < data < \infty$
Less expressed than GAPDH	*Same expression than GAPDH*	*More expressed than GAPDH*

Assumption with ΔC_T methods:

The ΔC_T method assumes an amplification efficiency of 1, however here again the method is considered as applicable for efficiency above 0.9. Moreover the error can be calculated using equation 9.

3.4. Primer Pair Validation

Primers validation is necessary to ensure that the correct cDNA product is amplified, that the fluorescence is due to the amplification of the target cDNA and is not due to mispriming or primer-dimer artifacts. The cycle at which the fluorescence is detected above the background is termed the threshold cycle (or C_T) and is used to determined transcript expression. Furthermore, during primer validation the primer pair efficiency E is calculated which is particularly important for the comparison of gene expression between samples. The threshold at which fluorescence is detected is also determined during primer validation.

3.4.1. Primer Sequences

Each primer pair was ordered from Invitrogen using sequences obtained from the PrimerBank database (Harvard), except primers for ITGA4 and ITGA6 genes which were order through QIAGEN (UK). The sequences were as follows:

Table 3 - Primer sequences used in quantitative PCR

Integrin Subunits or Markers	Gene names	Oligo Name	Oligo Sequence 5' to 3'	Amplicon length (bp)
α1	ITGA1	ITGA1 Fwd v2	CAGCCCCACATTTCAAGTCGT	174
		ITGA1 Rev v2	ACCTGTGTCTGTTTAGGACCA	
α2	ITGA2	ITGA2 Fwd v1	GCAACTGGTTACTGGTTGGTT	167
		ITGA2 Rev v1	GAGGCTCATGTTGGTTTTCATCT	
α3	ITGA3	ITGA3 Fwd v3	TCAACCTGGATACCCGATTCC	93
		ITGA3 Rev v3	GCTCTGTCTGCCGATGGAG	
α4	ITGA4	ITGA4 QIAGEN	unknown	70
			unknown	
α5	ITGA5	ITGA5 Fwd v1	GCCTGTGGAGTACAAGTCCTT	163
		ITGA5 Rev v1	AATTCGGGTGAAGTTATCTGTGG	
α6	ITGA6	ITGA6 QIAGEN	unknown	142
			unknown	
α7	ITGA7	ITGA7 Fwd v2	TGACCAACATTGATAGCTCAGAC	212
		ITGA7 Rev v2	GCGCAGGATAACCACAGCA	
α8	ITGA8	ITGA8 Fwd v1	ATGCCGAGTTCTCTCCTTGC	110
		ITGA8 Rev v1	TCCCACAATAAGGTCTCCATTCT	
α9	ITGA9	ITGA9 Fwd v1	AAGGAATTGCCGTTCAGAGGA	159
		ITGA9 Rev v1	TCATAGGCATCATCTCCGAGG	

α10	ITGA10	ITGA10 Fwd v1	CTTCAGTTCTGGGATATGTGCC	167
		ITGA10 Rev v1	CCAGTCTTCGTAGGAAGGTCT	
α11	ITGA11	ITGA11 Fwd v2	TCACGGACACCTTCAACATGG	114
		ITGA11 Rev v2	CCAGCCACTTATTGCCACTGA	
αV	ITGAV	ITGAV Fwd v1	CATCTGTGAGGTCGAAACAGG	137
		ITGAV Rev v1	TGGAGCATACTCAACAGTCTTTG	
β1	ITGB1	ITGB1 Fwd v2	TTATTGGCCTTGCATTACTGCT	147
		ITGB1 Rev v2	CCACAGTTGTTACGGCACTCT	
β2	ITGB2	ITGB2 Fwd v1	CAACGTATGCGAGTGCCATTC	180
		ITGB2 Rev v1	TTCACGGGGTTGTTCGACAG	
β3	ITGB3	ITGB3 Fwd v1	AGAGCCAGAGTGTCCCAAG	202
		ITGB3 Rev v1	GGCCTCTTTATACAGTGGGTTGT	
β5	ITGB5	ITGB5 Fwd v2	CAGGTGGAGGACTATCCTGTG	190
		ITGB5 Rev v2	GTGCCGTGTAGGAGAAAGGAG	
β6	ITGB6	ITGB6 Fwd v1	CATGTCCGCCAGACTGAGG	101
		ITGB6 Rev v1	GAGCCCAGCTCCTTTATTGTG	
β7	ITGB7	ITGB7 Fwd v1	TGGTTTTGGTTCCTTTGTGGA	119
		ITGB7 Rev v1	GGTGAAAGCTGAATGGTGACT	
β8	ITGB8	ITGB8 Fwd v2	CAGCACTGTGTCAATTCAAAGG	132
		ITGB8 Rev v2	GCAGGCTGTATAACAGGTGGG	
Aggrecan	ACAN	ACAN Fwd v1	ATTGGTGCCAAAAAGGATCAGT	101
		ACAN Rev v1	AGGATTGCTTCGAGAAAAACCAG	
Collagen I	COL1A1	COL1A1 Fwd v1	GTCGAGGGCCAAGACGAAG	143
		COL1A1 Rev v1	CAGATCACGTCATCGCACAAC	
Collagen II	COL1A1	COL2A1 Fwd v1	GGTCTTGGTGGAAACTTTGCT	114
		COL2A1 Rev v1	GGTCCTTGCATTACTCCCAAC	
Collagen X	COL10A1	COL10A1 Fwd v1	ATGCTGCCACAAATACCCTTT	134
		COL10A1 Rev v1	GGAATGAAGAACTGTGTCTTGGT	
GAPDH	GAPDH	GAPDH Fwd v1	ATGGGGAAGGTGAAGGTCG	150
		GAPDH Rev v1	GGGGTCATTGATGGCAACAATA	
PPARγ	PPARG	PPARG Fwd v1	CCTATTGACCCAGAAAGCGATT	135
		PPARG Rev v1	CATTACGGAGAGATCCACGGA	
RunX2	RUNX2	RUNX2 Fwd v1	TCCTATGACCAGTCTTACCCCT	190
		RUNX2 Rev v1	GGCTCTTCTTACTGAGAGTGGAA	
Sox9	SOX9	SOX9 Fwd v1	AGCGAACGCACATCAAGAC	110
		SOX9 Rev v1	GCTGTAGTGTGGGAGGTTGAA	

3.4.2. Primer Pairs Validation with Quantitative Polymerase Chain Reaction (qPCR) and Melt Curve Analysis

A serial dilution of cDNA from hMSC culture on tissue culture plastic was prepared. In order to get a standard curve, five dilutions of cDNA were amplified: 1:10, 1:50, 1:100, 1:1000, and 1:10,000. qPCR was performed as described in Section 3.3.2. Each real-time PCR was carried out in technical triplicates. For the same primer set, different threshold cycles (C_T) were obtained.

The C_T obtained were plotted against dilutions on a logarithmic scale (log_{10}) and once inaccurate replicates were removed the standard curve was calculated using Rotor-Gene ScreenClust Software (QIAGEN, UK). Technical replicates were considered as inaccurate when C_T differences between them exceeded half a cycle.

During primer validation, the primer pair efficiency was calculated, the fit of the standard curve was also assessed and most importantly the threshold at with fluorescence is detected was determined.

The amplification curves were characterised by four phases: the background, the exponential phase, the linear phase and eventually the plateau. The threshold has to be in the exponential phase, and should be the lowest possible.[73]

The efficiency of the pair primers is given by the slope of the standard curve and ideally equals 1 when slope is -3.32 meaning that the target cDNA is exactly doubled at each PCR cycle: $slope = \frac{\Delta y}{\Delta x} = \frac{1\,Cycle}{log_{10}(2)} = -3.32$. Primer pairs were considered validated when their amplification efficiency was above 0.9 and the fit of the curve which was expressed by R^2 above 0.99.

Moreover the melt curve was verified for each primer pair. As the melting temperature of a cDNA double helix depends on its base composition and its length it characterises the cDNA sequence and thus only one melting temperature should be identified: the one of the amplified target cDNA. The temperature was raised stepwise from 70 to 95°C, starting with 90 s of pre-melt conditioning for the first step the temperature was then raised at 1°C at each step, the duration of each step was 5 s. At each step the change in fluorescence was measured, the melt curve plots the negative derivative with respect to time of the fluorescence against the temperature, the peak of the derivative is reached when the cDNA double helix is separated which occurs at the melting temperature.

3.4.3. Assessing Primers by Gel Electrophoresis

Analysis of the melt curve ensured a single product was being amplified, but in order to confirm that it was the correct amplicon, we performed agarose gel DNA electrophoresis. A 2% gel was appropriate for these DNA fragment sizes. Briefly, 2 g of agarose was added to 100 ml 1X TAE buffer and dissolved by heating up until reaching boiling temperature. Once cooled down, 10 µl of SYBR Safe (Invitrogen, UK) was added. The gel was then poured in the cast and left at room temperature until set. 2 µl of 6X loading buffer (Promega, UK) was added to 10 µl of sample and a final volume of 12 µl was loaded per well. The gel was run for 90 min at 100-120 V and bands were visualised using an UV illumination.

3.5. Histology

The same procedure for histology was applied to pellet culture, micromass culture and collagen II hydrogels. Samples were harversted on day 21, fixed with 3.7 % (w/v) paraformaldehyde in PBS for 20 min, washed twice in PBS, and stored at 4°C in until being sent for analysis at the histology facility of Imperial College London. Hematoxylin and eosin (H&E) staining was performed on the samples to evaluate cell morphology, where the hematoxylin stains nuclei in blue while eosin stains mostly the cytoplasm in red. Sulphated glycosaminoglycans (GAGs) were visualised with alcian blue staining and collagen was detected by picosirius red staining.

3.6. Statistical Analysis

The Student *t*-test was applied in order to determine whether the difference between samples was significant, all tests were two-tailored. Two-way ANOVA Analysis of data was also performed on data using SigmaPlot version 12. The results were considered significantly different at a P-value of less or equal 0.05.

4. Results

4.1. Differentiation Efficiency

Chondrogenic differentiation was assessed in pellet culture, micromass culture and 3D collagen II gels by quantifying the transcript levels of the following chondrocyte phenotypes markers: ACAN, SOX9, COL1A1, COL2A1 and COL10A1. We also investigated expression of PPARγ and RUNX2 to ensure that the hMSCs were not committed into adipogenic or osteogenic lineage, respectively. Additionally, as gene expression alone is not sufficient to assess chondrogenesis, we confirmed differentiation with histological staining for GAGs and collagen deposition after 21 days in culture.

4.1.1. Transcript Levels of Phenotype Markers in Pellet Culture, Micromass Culture and Collagen II 3D Hydrogels

Pellet culture:

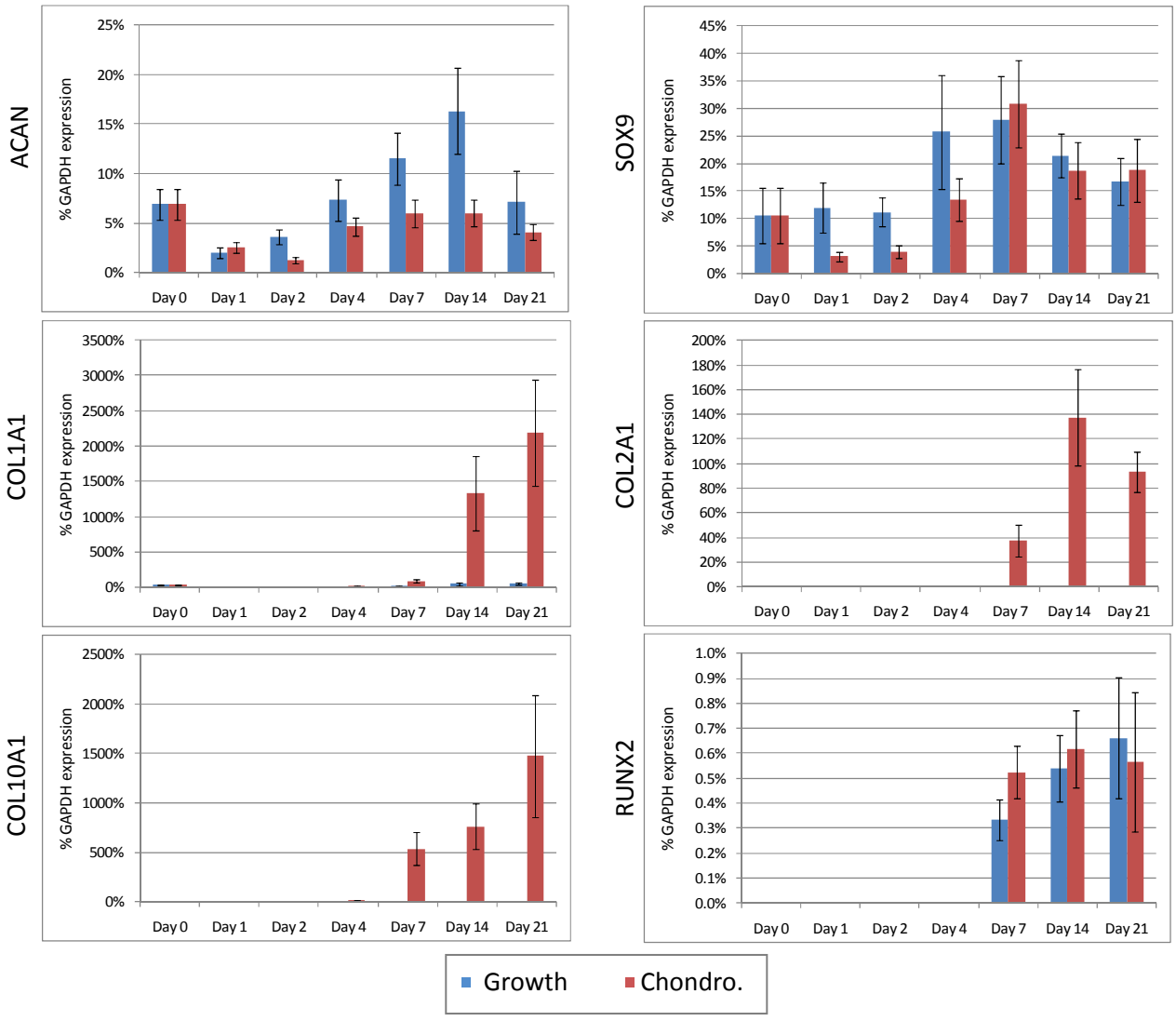


Figure 9 - Quantitative analysis of several transcripts in hMSCs cultured in pellet culture up to 21 days in either growth or chondrogenic medium. The expression level was normalised such that the level of GAPDH expression was 100%. Bars represent the mean ± standard error from three independent experiments performed in technical triplicate.

The expression of *ACAN* remained relatively constant throughout chondrogenesis, while *SOX9* expression almost doubled, however in both cases no significant differences were observed between pellet in the growth medium and chondrogenic medium (***Figure 9***). Bosnakovski *et al*. have reported that in 3D culture systems *SOX9* and *ACAN* expressions were upregulated indifferently to the medium (growth or chondrogenic medium).[70] *PPAR*γ, an adipocyte lineage marker, was not detected at any time and *RUNX2* expression, a bone lineage marker, was detected from Day 7 onwards, both in control and chondrogenic pellets, however its expression level remained very low. Importantly collagen type II was detected from Day 7 only in the pellets in chondrogenic medium and reached high level of expression, up to 1.4 fold highly than *GAPDH*. *COLA1A1* and *COL10A1* were also expressed at very high levels from Day 7 onwards, up to 22-times and 15-times *GAPDH* expression respectively. From these data, the hMSCs have undergone chondrogenesis as shown by the upregulation of *SOX9* and all three Collagen types, however the cells tend to upregulate genes associated with fibrocartilage ECM rather than hyaline cartilage as shown by the very high expression of *COL1A1*. Moreover the upregulation of *COL10A1* and *RUNX2* at late time points suggest that some hMSCs might eventually undergo hypertrophy and that few cells might not be fully committed to chondrogenic lineage but instead are undergoing osteogenesis.

Micromass culture:

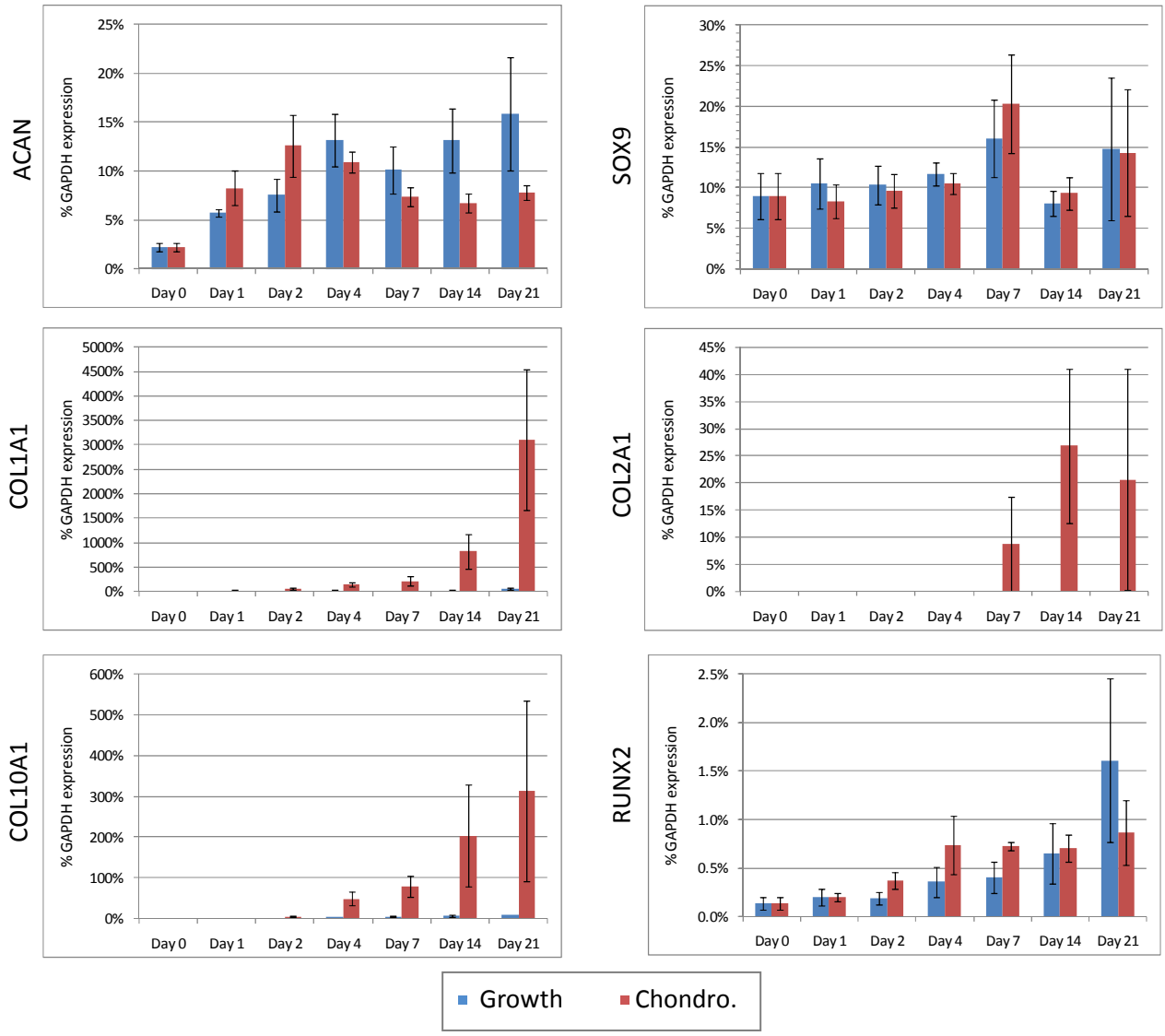


Figure 10 - Quantitative analysis of several transcripts in hMSCs cultured in micromass culture up to 21 days in either growth or chondrogenic medium. The expression level was normalised such that the level of GAPDH expression was 100%. Bars represent the mean ± standard error from three independent experiments performed in technical triplicate (with the exception of Day 21 where N=2).

In micromass cultures, *ACAN* was significantly upregulated in micromass cultures in growth and chondrogenic medium but no significant differences were noticeable between these two culture conditions, which could be explain by the 3D environment (***Figure 10***).[70] Particularly, the upregulation of *ACAN* is significant between Day 0 and Day 21 of chondrogenic micromasses ($p < 0.05$). SOX9 remains constant throughout the differentiation process and no significant differences were observed between growth and chondrogenic micromass culture. *PPARγ* was never detected and *RUNX2* expression was detected on Day 0 and was slightly upregulated, but this variation was not significant, and moreover no differences were observed between control and chondrogenic conditions. Concerning the different collagens, collagen type II expression was detectable from Day 7 onwards only in the cells grown in chondrogenic medium, where its expression level only reached a

25% of *GAPDH* levels. Importantly *COL2A1* was not detected in the control cells. *COL1A1* expression was detected on Day 0 at low levels, a 30-fold increase was detected throughout the chondrogenesis for micromass cultured in growth and an 1800-fold increase was detected when cultured in chondrogenic medium or 300-time fold *GAPDH* expression. Similarly for *COL10A1* expression a 37-fold change was measured between Day 0 and Day 21 and a fold increase over 1300 was measured between Day 0 and Day 21 for control and chondrogenic micromass cultures respectively.

Collagen type II 3D hydrogels:

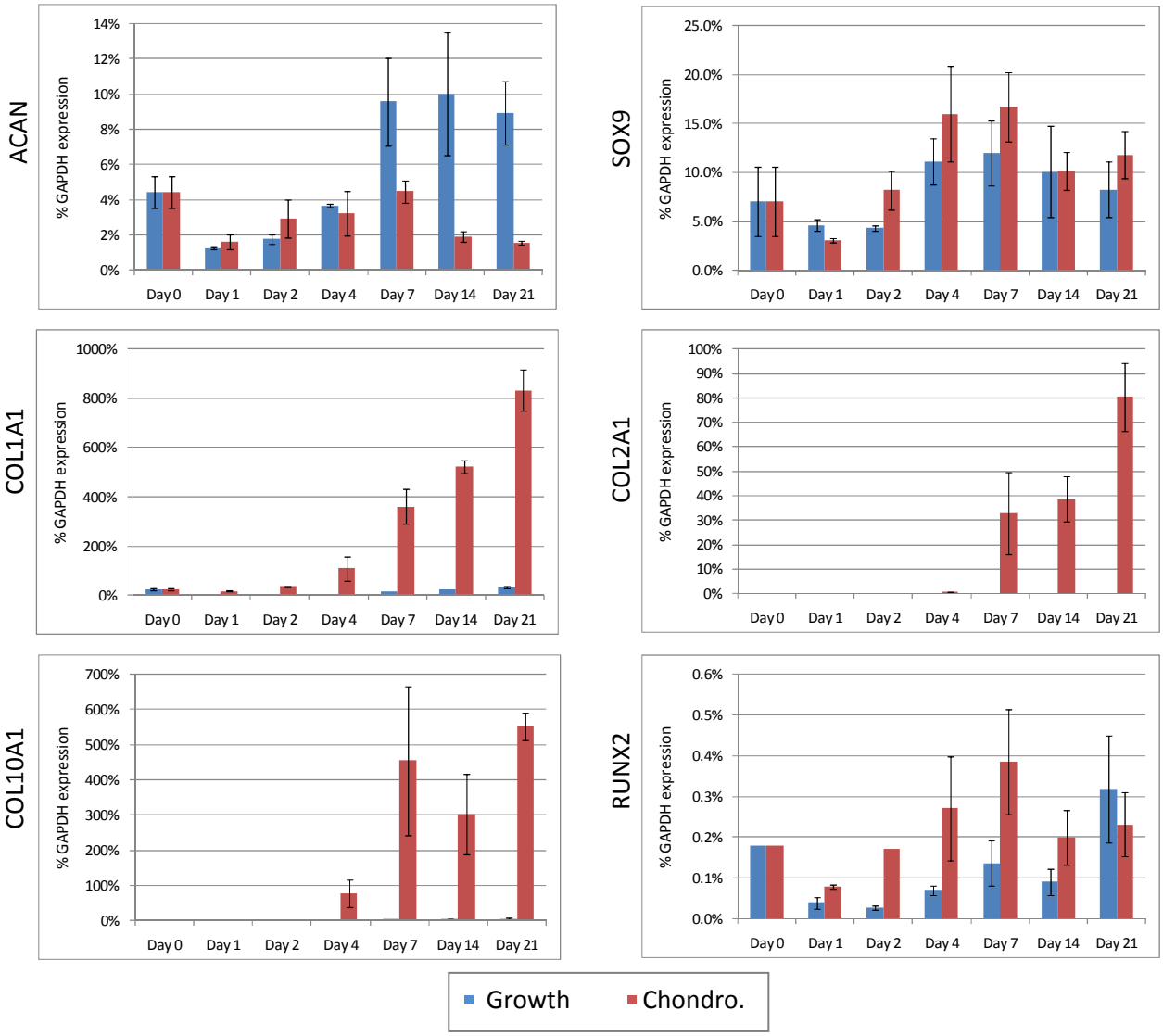


Figure 11 - Quantitative analysis of several transcripts in hMSCs cultured in pellet culture up to 21 days in either growth or chondrogenic medium. The expression level was normalised such that the level of GAPDH expression was 100%. Bars represent the mean ± standard error from three independent experiments performed in technical triplicate. (Few time points N=2).

ACAN was unexpectedly upregulated in gels cultured in growth medium, while downregulated in cells culture in chondrogenic medium, however these variations are not statistically significant (***Figure 11***). Similarly, *SOX9* expression remained constant with no significant changes throughout the differentiation between gels cultured in chondrogenic medium and control gels. *PPARγ* was not detected and *RUNX2* expression was detected throughout the differentiation process in both conditions however its variations were not significant. *COL2A1* expression was switch on only in cells cultured in chondrogenic medium and gradually increased to reach 80% of *GAPDH* expression level on Day 21. *COL1A1* expression was detected on Day 0 and was continuously upregulated in chondrogenic culture conditions, from Day 7 onwards the expression increase was statistically significant, while expression remained constant in control conditions. *COL10A1* was detected from Day 4 onwards, its expression significantly increased in chondrogenic conditions but remained constant in control culture conditions.

4.1.2. Histology in Pellet Culture, Micromass Culture and 3D Collagen II Hydrogels

Pellet culture:

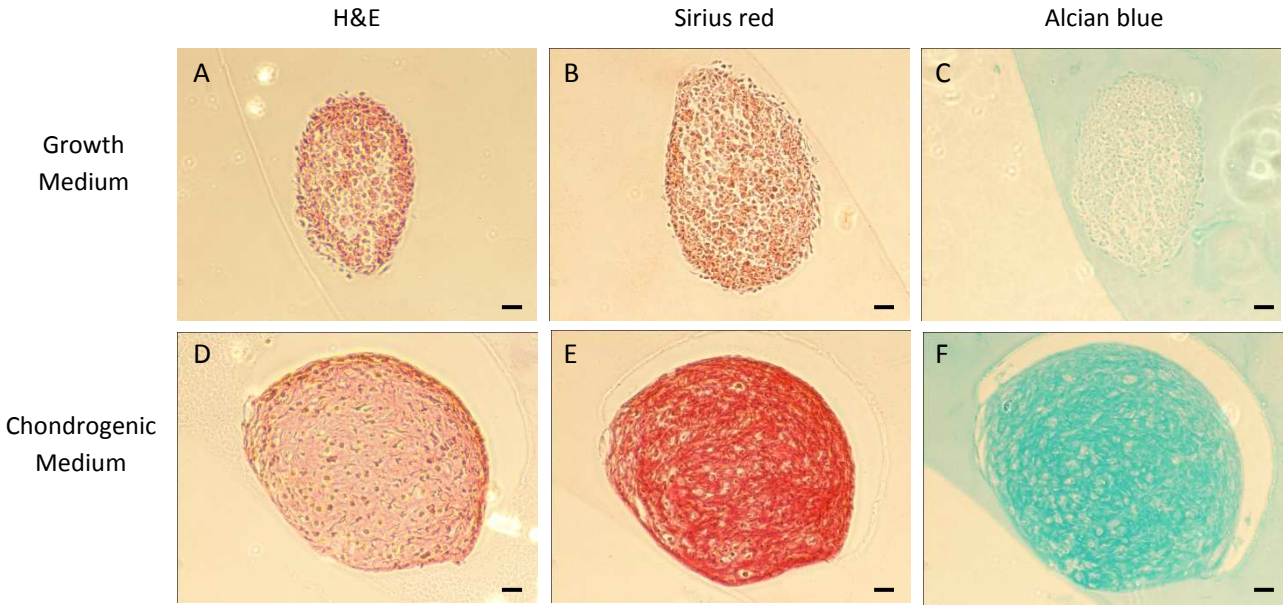


Figure 12 – Histology in pellet culture. Cells cultured in pellet were fixed on Day 21 and stained with hematoxylin and eosin (A, D), for cytoplasm and nucleus staining, sirius red (B, E) to detect collagen deposition and alcian blue (C, F) for proteoglycans deposition. Scale bar 50 µm.

Pellets grown in chondrogenic medium were strongly stained with both staining sirius red and alcian blue compared to pellets grown in control medium, showing an important accumulation of both GAGs and collagen as expected after chondrogenesis (***Figure 12***).

Micromass culture:

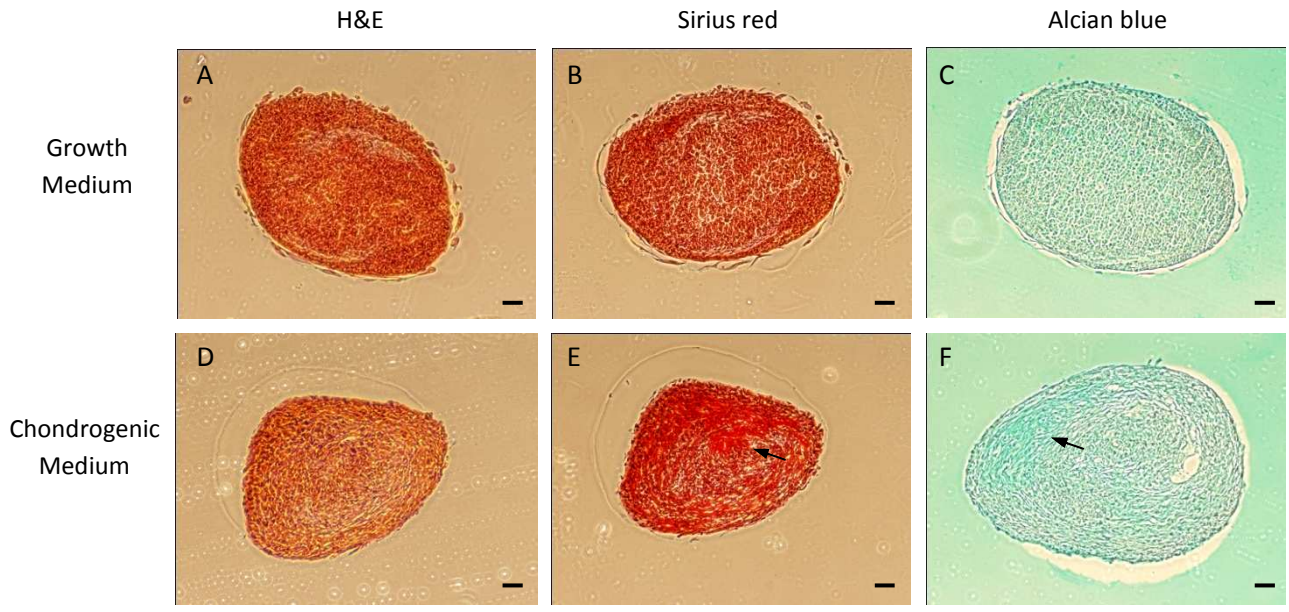


Figure 13 - Histology in micromass culture. Cells cultured in pellet were fixed on Day 21 and stained with hematoxylin and eosin (A, D), for cytoplasm and nucleus staining, sirius red (B, E) to detect collagen deposition and alcian blue (C, F) for proteoglycans deposition. Scale bar 50 µm.

Accumulation of collagens was shown in micromass cultures by using Sirius red, micromass cultured in chondrogenic medium were positively stained yet weakly, however no staining was observed in the control micromass culture (***Figure 13***). The alcian blue staining was weak in chondrogenic conditions and absent in control conditions.

Collagen type II gels:

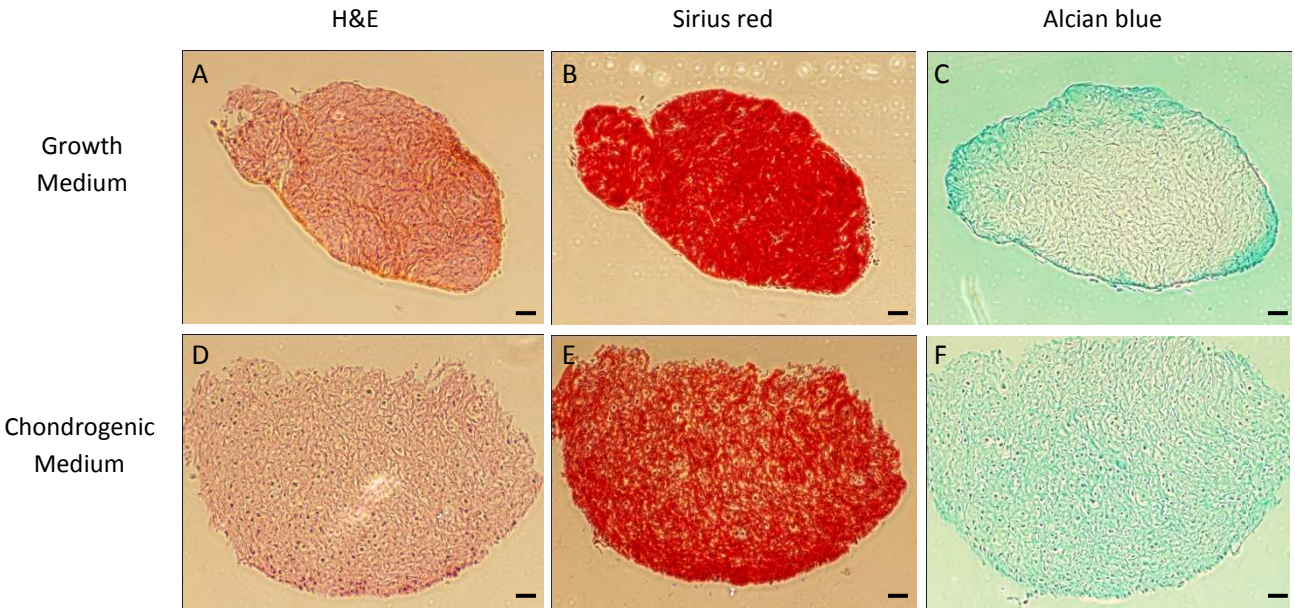


Figure 14 - Histology in collagen type II gels. Cells cultured in pellet were fixed on Day 21 and stained with hematoxylin and eosin (A, D), for cytoplasm and nucleus staining, sirius red (B, E) to detect collagen deposition and alcian blue (C, F) for proteoglycans deposition. Scale bar 50 µm.

The gels cultured in growth and chondrogenic medium were both strongly stained for collagen with Sirius red as the gels are made of collagen, no differences were expected between these two conditions and a strong staining is consequently normal (***Figure 14***). Concerning PGs staining, in control conditions the staining was clearly negative while in chondrogenic medium we had a weak alcian blue staining confirming PGs deposition.

4.2. Integrin expression

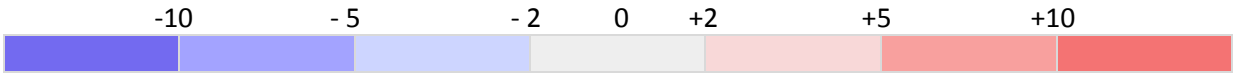
The expression of each integrin subunit listed in ***Table 2*** were analysed at least once throughout the 21-day experiment for the three different culture methods which are pellet culture, micromass culture and collagen type II 3D gels. The integrin subunits having a low and particularly irregular expression pattern and subunits that were not expressed were dropped at this point of the experiment. We carried on with the following subunits (***Table 4***):

Table 4 - Integrin subunits investigated in the present study

	Alpha subunits	Beta subunits
Subunits studied throughout the 21 days-experiment	ITGA1, ITGA2, ITGA3, ITGA5, ITGA6, ITGA6, ITGA7, ITGA10, ITGA11 and ITGAV	ITGB1, ITGB3, ITGB5 and ITGB8
Subunits discarded	ITGB8, ITGB9, ITGAD, ITGAE, ITGAL, ITGAM, ITGAW and ITGAX	ITGB2, ITGB4, ITGB6 and ITGB7

The investigated integrin subunits expression levels are relative to the expression in untreated hMSCs cultured on tissue culture plastic, a colour legend according to the fold-change was assigned to the integrin transcript expression levels. The data are summarised in the following tables as the mean value ± standard error (***Table 5, 6, and 7***).

Legend: fold change compare to untreated hMSC cultured on tissue culture plastic.



4.2.1. Pellet Culture

Table 5 - Integrin transcript expression in pellet culture in either growth medium (A) or chondrogenic medium (B). The expression level was quantified by qPCR. Data are shown as mean ± standard error from three independent experiments performed in technical triplicate.

A

	Pellet Cultures – Growth medium						
	Day 0	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**Day 14**	**Day 21**
ITGA1	1.85 ± 0.32	1.33 ± 0.28	1.48 ± 0.33	4.56 ± 1.41	6.94 ± 2.40	18.93 ± 4.81	11.46 ± 3.28
ITGA2	2.06 ± 0.93	1.97 ± 0.44	2.41 ± 1.05	4.80 ± 0.83	4.69 ± 0.96	3.30 ± 1.03	2.28 ± 0.63
ITGA3	2.55 ± 0.41	-1.52 ± 0.35	-1.74 ± 0.20	1.15 ± 0.09	1.56 ± 0.30	2.28 ± 0.56	2.29 ± 0.59
ITGA4	1.40 ± 0.27	-2.92 ± 0.49	-2.74 ± 1.45	-1.67 ± 0.47	-1.34 ± 0.47	1.36 ± 0.37	-1.36 ± 0.35
ITGA5	1.11 ± 0.32	1.04 ± 0.15	-1.15 ± 0.07	-1.06 ± 0.29	-1.16 ± 0.23	-1.55 ± 0.30	-1.50 ± 0.30
ITGA6	1.82 ± 0.32	-3.91 ± 1.19	-4.27 ± 0.89	-1.58 ± 0.26	-1.76 ± 0.57	-2.37 ± 0.14	-2.43 ± 1.12
ITGA7	6.75 ± 1.79	1.34 ± 0.35	-1.08 ± 0.09	2.35 ± 0.46	3.77 ± 1.51	11.94 ± 4.16	4.74 ± 1.91
ITGA10	7.42 ± 1.81	2.01 ± 0.87	1.49 ± 0.40	4.62 ± 0.57	24.76 ± 8.72	30.57 ± 9.31	21.97 ± 6.08
ITGA11	2.37 ± 0.28	-1.23 ± 0.19	-1.66 ± 0.25	1.72 ± 0.28	5.62 ± 2.18	8.76 ± 2.16	10.36 ± 2.61
ITGAV	1.87 ± 0.55	-1.93 ± 0.54	-1.86 ± 0.25	-1.16 ± 0.20	2.14 ± 0.54	1.95 ± 0.35	1.83 ± 0.59
ITGB1	1.72 ± 0.48	-2.35 ± 0.48	-2.38 ± 0.70	-1.38 ± 0.38	1.39 ± 0.34	1.77 ± 0.35	1.73 ± 0.40
ITGB3	1.78 ± 0.46	1.09 ± 0.30	1.86 ± 0.52	2.30 ± 0.82	1.03 ± 0.31	1.24 ± 0.13	1.53 ± 0.36
ITGB5	1.09 ± 0.15	-2.33 ± 0.44	-2.09 ± 0.41	1.30 ± 0.17	4.23 ± 1.12	5.70 ± 1.31	4.26 ± 1.06
ITGB8	3.52 ± 0.83	2.21 ± 0.53	6.94 ± 1.78	11.81 ± 3.35	31.15 ± 7.89	38.65 ± 6.76	27.71 ± 11.56

B

	Pellet cultures – Chondrogenic medium						
	Day 0	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**Day 14**	**Day 21**
ITGA1	1.85 ± 0.32	1.38 ± 0.12	1.63 ± 0.57	2.07 ± 0.43	4.08 ± 1.25	4.82 ± 1.90	2.74 ± 0.98
ITGA2	2.06 ± 0.93	-2.84 ± 0.38	-3.40 ± 0.07	-2.84 ± 0.39	-2.35 ± 0.47	-6.23 ± 2.19	-16.07
ITGA3	2.55 ± 0.41	-1.30 ± 0.72	-5.78 ± 1.09	-6.74 ± 1.04	-3.60 ± 1.30	-16.20 ± 7.59	-15.91 ± 4.23
ITGA4	1.40 ± 0.27	-8.58 ± 1.93	-10.47 ± 0.52	-4.58 ± 1.11	-2.63 ± 0.57	-2.48 ± 1.13	-5.85 ± 1.73
ITGA5	1.11 ± 0.32	1.30 ± 0.12	-1.42 ± 0.22	-1.78 ± 0.2	-2.19 ± 0.39	-4.79 ± 0.89	-5.80 ± 1.10
ITGA6	1.82 ± 0.32	-12.79 ± 0.80	-20.31 ± 8.31	-7.89 ± 3.62	-5.21 ± 1.49	-9.53 ± 4.95	-10.07 ± 4.51
ITGA7	6.75 ± 1.79	-1.00 ± 0.31	-1.57 ± 0.16	2.19 ± 0.73	3.35 ± 0.24	9.01 ± 3.98	2.11 ± 0.3
ITGA10	7.42 ± 1.81	2.80 ± 0.20	1.88 ± 0.52	3.81 ± 1.20	8.60 ± 1.96	6.15 ± 2.39	6.43 ± 2.01
ITGA11	2.37 ± 0.28	2.03 ± 0.97	1.71 ± 0.90	2.37 ± 0.79	6.43 ± 1.45	13.50 ± 3.98	14.46 ± 1.56
ITGAV	1.87 ± 0.55	-1.46 ± 0.62	-1.11 ± 0.30	3.13 ± 0.55	4.81 ± 1.23	3.23 ± 0.81	2.74 ± 0.68
ITGB1	1.72 ± 0.48	-2.40 ± 0.60	-2.59 ± 0.60	-2.00 ± 0.53	-1.04 ± 0.26	1.13 ± 0.34	1.08 ± 0.32
ITGB3	1.78 ± 0.46	-1.65 ± 0.38	-2.40 ± 0.88	-2.26 ± 0.59	-1.44 ± 0.59	-17.40	-7.98 ± 2.86
ITGB5	1.09 ± 0.15	-3.17 ± 1.03	-3.89 ± 1.16	-2.60 ± 0.65	-1.23 ± 0.29	-1.06 ± 0.30	1.45 ± 0.30
ITGB8	3.52 ± 0.83	-1.24 ± 0.46	1.59 ± 0.84	3.74 ± 1.11	4.53 ± 0.89	2.01 ± 0.61	2.44 ± 1.23

4.2.2. Micromass Culture

Table 6 - Integrin transcript expression in micromass culture in either growth medium (A) or chondrogenic medium (B). The expression level was quantified by qPCR. Datas are shown as mean ± standard error from three independent experiments performed in technical

A

	Micromass Cultures – Growth medium						
	Day 0	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**Day 14**	**Day 21**
ITGA1	1.25 ± 0.35	3.88 ± 0.67	5.96 ± 1.85	17.07 ± 5.69	18.88 ± 3.91	20.36 ± 9.83	18.55 ± 11.41
ITGA2	1.39 ± 0.45	3.15 ± 1.04	2.63 ± 1.03	3.82 ± 1.30	3.57 ± 1.13	2.12 ± 0.83	1.22 ± 0.68
ITGA3	-5.55 ± 1.92	-2.28 ± 0.84	-2.05 ± 0.86	1.02 ± 0.41	1.14 ± 0.41	-1.25 ± 0.30	1.52 ± 0.87
ITGA4	-4.07 ± 0.82	-2.31 ± 0.24	-2.82 ± 0.44	-1.28 ± 0.14	-1.29 ± 0.63	-1.30 ± 0.77	-1.68 ± 1.20
ITGA5	-1.70 ± 0.24	-1.55 ± 0.27	-1.62 ± 0.30	-1.86 ± 0.34	-2.29 ± 0.67	-3.32 ± 0.59	2.65 ± 1.44
ITGA6	-15.01 ± 5.19	-9.18 ± 4.12	-8.47 ± 3.59	-4.53 ± 2.05	-3.97 ± 0.85	-3.33 ± 2.13	-3.60 ± 2.30
ITGA7	-1.34 ± 5.19	3.22 ± 1.44	2.89 ± 1.87	8.97 ± 6.15	3.81 ± 1.31	3.09 ± 1.84	3.49 ± 2.20
ITGA10	1.27 ± 0.54	3.43 ± 1.26	7.74 ± 4.86	25.25 ± 16.05	20.38 ± 8.68	18.72 ± 7.34	16.02 ± 9.04
ITGA11	-2.41 ± 0.70	-1.10 ± 0.15	1.12 ± 0.45	3.00 ± 0.60	5.76 ± 2.31	4.71 ± 1.86	6.36 ± 3.10
ITGAV	-4.23 ± 0.67	-1.44 ± 0.18	-1.06 ± 0.34	2.07 ± 0.52	2.10 ± 0.36	2.45 ± 0.52	1.83 ± 0.81
ITGB1	-3.94 ± 1.04	-1.74 ± 0.47	-1.91 ± 0.55	-1.04 ± 0.23	2.19 ± 0.83	3.18 ± 1.36	2.87 ± 1.25
ITGB3	-1.20 ± 0.19	1.59 ± 0.27	1.49 ± 0.14	1.47 ± 0.31	-1.21 ± 0.20	1.28 ± 0.12	1.25 ± 0.67
ITGB5	-1.54 ± 0.36	1.81 ± 0.24	2.24 ± 0.59	4.66 ± 1.11	5.52 ± 1.50	5.68 ± 1.36	6.23 ± 2.63
ITGB8	3.93 ± 0.27	6.98 ± 0.72	15.13 ± 1.75	20.36 ± 5.16	24.35 ± 6.80	34.26 ± 12.36	52.47 ± 23.69

B

	Micromass cultures – Chondrogenic medium						
	Day 0	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**Day 14**	**Day 21**
ITGA1	1.25 ± 0.35	3.61 ± 1.39	5.16 ± 1.68	5.93 ± 2.45	4.83 ± 1.69	5.07 ± 2.49	5.16 ± 2.54
ITGA2	1.39 ± 0.45	2.81 ± 1.12	2.78 ± 0.92	1.96 ± 0.83	-1.83 ± 0.81	-3.31 ± 0.90	-16.89
ITGA3	-5.55 ± 1.92	-3.25 ± 1.04	-1.98 ± 0.75	-1.88 ± 0.63	-4.52 ± 0.82	-6.48 ± 1.09	-4.52 ± 3.06
ITGA4	-4.07 ± 0.82	-2.54 ± 1.19	-3.40 ± 1.35	-1.49 ± 0.73	-2.87 ± 0.78	-2.71 ± 0.99	-4.96
ITGA5	-1.70 ± 0.24	2.01 ± 0.34	2.32 ± 0.61	1.80 ± 0.57	-1.92 ± 0.65	-4.71 ± 0.38	-2.20 ± 1.27
ITGA6	-15.01 ± 5.19	-6.34 ± 1.94	-5.56 ± 2.17	-4.42 ± 2.97	-4.55 ± 2.68	-5.54 ± 4.06	-8.47 ± 6.28
ITGA7	-1.34 ± 5.19	8.41 ± 3.63	10.57 ± 6.96	13.82 ± 9.97	4.21 ± 1.82	3.39 ± 1.92	2.52 ± 1.78
ITGA10	1.27 ± 0.54	5.50 ± 2.39	11.75 ± 5.79	19.70 ± 10.88	12.24 ± 3.09	3.74 ± 0.21	6.97 ± 3.83
ITGA11	-2.41 ± 0.70	-1.11 ± 0.52	2.38 ± 0.91	7.48 ± 1.36	8.29 ± 2.56	7.93 ± 1.68	15.43 ± 8.35
ITGAV	-4.23 ± 0.67	2.99 ± 0.12	4.50 ± 0.98	7.12 ± 2.15	4.75 ± 1.06	3.20 ± 0.37	4.12 ± 1.90
ITGB1	-3.94 ± 1.04	-1.16 ± 0.39	1.38 ± 0.36	1.98 ± 0.34	3.19 ± 1.52	3.33 ± 1.67	4.89 ± 2.40
ITGB3	-1.20 ± 0.19	1.21 ± 0.32	1.85 ± 0.88	-1.07 ± 0.61	-2.92 ± 1.36	-5.98	3.92
ITGB5	-1.54 ± 0.36	1.59 ± 0.29	2.07 ± 0.42	3.01 ± 0.52	2.16 ± 0.23	1.79 ± 0.32	3.40 ± 1.43
ITGB8	3.93 ± 0.27	5.72 ± 1.44	10.58 ± 3.36	10.12 ± 4.58	9.54 ± 1.37	12.02 ± 2.59	12.05 ± 7.25

4.2.3. Collagen II 3D Gels

Table 7 - Integrin transcript expression in collagen type II gels in either growth medium (A) or chondrogenic medium (B). The expression level was quantified by qPCR. Datas are shown as mean ± standard error from three independent experiments performed in technical. (For some time points N=1 or 0, orange cases).

A

	Collagen type II 3D gels – Growth medium						
	Day 0	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**Day 14**	**Day 21**
ITGA1	1.04 ± 0.08	-1.43 ± 0.21	-1.30 ± 0.28	4.94 ± 1.77	13.07 ± 1.30	12.26 ± 3.81	14.42 ± 4.22
ITGA2	-1.76 ± 0.48	1.07 ± 0.09	1.36 ± 0.03	1.28 ± 0.13	4.85 ± 1.46	4.53 ± 1.56	3.07 ± 0.98
ITGA3	-2.06 ± 0.99	-8.40 ± 2.29	-9.72 ± 1.84	-5.81 ± 2.59	-1.63 ± 0.32	-1.34 ± 0.19	-1.17 ± 0.21
ITGA4	1.85 ± 0.05	-5.60 ± 0.67	-5.39 ± 1.47	-2.52 ± 0.06	1.54 ± 0.08	1.60 ± 0.21	1.22 ± 0.14
ITGA5	-1.73 ± 0.18	-1.88 ± 0.17	-3.02 ± 0.18	-2.63 ± 0.12	-2.17 ± 0.66	-2.21 ± 0.68	-3.86 ± 0.64
ITGA6	-1.04 ± 0.45	-8.86 ± 3.00	-15.81 ± 5.30	-6.56 ± 3.07	-1.90 ± 0.85	-2.16 ± 0.83	-3.85 ± 1.47
ITGA7	-1.27 ± 0.25	-3.32 ± 1.52	-4.85	-1.80 ± 0.76	-1.49 ± 0.27	-2.33 ± 0.99	-2.18 ± 0.80
ITGA10	2.09 ± 0.09	-2.48 ± 0.34	-2.65 ± 0.26	1.52 ± 0.15	9.52 ± 0.66	11.45 ± 2.48	9.59 ± 3.09
ITGA11	-2.15 ± 0.91	-4.42 ± 0.58	-5.28 ± 2.25	-1.93 ± 0.75	2.17 ± 0.37	2.33 ± 0.36	4.09 ± 1.67
ITGAV	1.17 ± 0.51	-2.94 ± 1.15	-4.87 ± 2.27	-3.61 ± 1.49	1.19 ± 0.39	-1.24 ± 0.27	1.18 ± 0.26
ITGB1	1.17 ± 0.10	-3.03 ± 0.14	-4.77 ± 0.18	-2.96 ± 0.07	1.23 ± 0.07	1.28 ± 0.25	1.14 ± 0.16
ITGB3	1.01 ± 0.07	-1.04 ± 0.44	-1.04 ± 0.17	1.22 ± 0.25	-1.13 ± 0.19	1.22 ± 0.30	1.49 ± 0.47
ITGB5	1.94 ± 1.08	-2.48 ± 0.58	-3.10 ± 0.41	-1.43 ± 0.20	3.26 ± 0.57	2.88 ± 0.66	3.48 ± 0.93
ITGB8	13.45 ± 11.09	-1.49 ± 0.21	1.64 ± 0.43	7.51 ± 0.26	23.89 ± 8.48	21.76 ± 5.44	23.65 ± 6.06

B

	Collagen type II gels – Chondrogenic medium						
	Day 0	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**Day 14**	**Day 21**
ITGA1	1.04 ± 0.08	-1.23 ± 0.10	1.78 ± 0.79	2.75 ± 0.75	3.19 ± 0.66	1.35 ± 0.24	1.06 ± 0.16
ITGA2	-1.76 ± 0.48	-3.01 ± 1.01	-2.32 ± 0.96	-1.68 ± 0.72	-3.07 ± 1.00	-14.33 ± 6.78	-36.17
ITGA3	-2.06 ± 0.99	-14.11 ± 5.41	-14.17 ± 4.54	-5.60 ± 1.15	-8.70 ± 3.34	-10.36	-8.91 ± 4.13
ITGA4	1.85 ± 0.05	-7.78 ± 1.16	-7.73 ± 1.45	-2.29 ± 0.92	-1.51 ± 0.24	-4.04 ± 1.65	-2.70 ± 0.59
ITGA5	-1.73 ± 0.18	-1.12 ± 0.17	-1.29 ± 0.26	-1.80 ± 0.48	-4.0 ± 0.58	-11.03 ± 1-41	-10.74 ± 1.49
ITGA6	-1.04 ± 0.45	-12.39 ± 3.43	-13.55 ± 5.57	-11.39 ± 8.28	-8.65 ± 2.54	TBD	TBD
ITGA7	-1.27 ± 0.25	-3.88 ± 1.66	-1.80 ± 0.11	1.42 ± 0.34	-1.38 ± 0.46	TBD	-7.24
ITGA10	2.09 ± 0.09	1.06 ± 0.28	1.37 ± 0.45	3.43 ± 1.59	2.65 ± 0.24	1.31 ± 0.16	2.38 ± 0.23
ITGA11	-2.15 ± 0.91	-2.07 ± 0.64	-1.32 ± 0.19	3.30 ± 1.01	3.04 ± 0.34	3.76 ± 0.76	4.51 ± 0.99
ITGAV	1.17 ± 0.51	1.35 ± 0.44	2.24 ± 0.17	2.47 ± 0.45	2.06 ± 0.20	1.27 ± 0.37	1.29 ± 0.49
ITGB1	1.17 ± 0.10	-2.76 ± 0.44	-1.97 ± 0.51	-1.24 ± 0.47	1.06 ± 0.15	-1.79 ± 0.07	-1.80 ± 0.16
ITGB3	1.01 ± 0.07	-2.56 ± 1.16	2.99 ± 1.37	-2.44	-2.32	-7.58	TBD
ITGB5	1.94 ± 1.08	-3.13 ± 0.83	-2.60 ± 0.83	-1.62 ± 0.65	-1.29 ± 0.22	-1.68 ± 0.18	-1.45 ± 0.15
ITGB8	13.45 ± 11.09	-1.42 ± 0.56	1.66 ± 0.62	4.46 ± 1.82	6.64 ± 1.69	8.22 ± 2.84	14.78 9.75

4.2.4. Expression of Alpha Integrin Subunits in the Different Culture Systems

ITGA1:

In the three culture methods, the ITGA1 expression remained constant in cells cultured in chondrogenic medium, while it was increased more than tenfold throughout the 21 days in growth medium (***Figure 15***). Moreover at the earliest time points, both control and chondrogenic conditions behave similarly in the three models. The expression pattern was significantly different over time between the growth and chondrogenic conditions in pellet culture and in collagen gels, but not in micromass due to high variability of ITGA1 expression in samples within the same group in expression levels. Finally, from Day 4 until the end of the experiment the expression of ITGA1 was always found in higher amount in cells cultured in growth medium than hMSCs undergoing chondrogenesis.

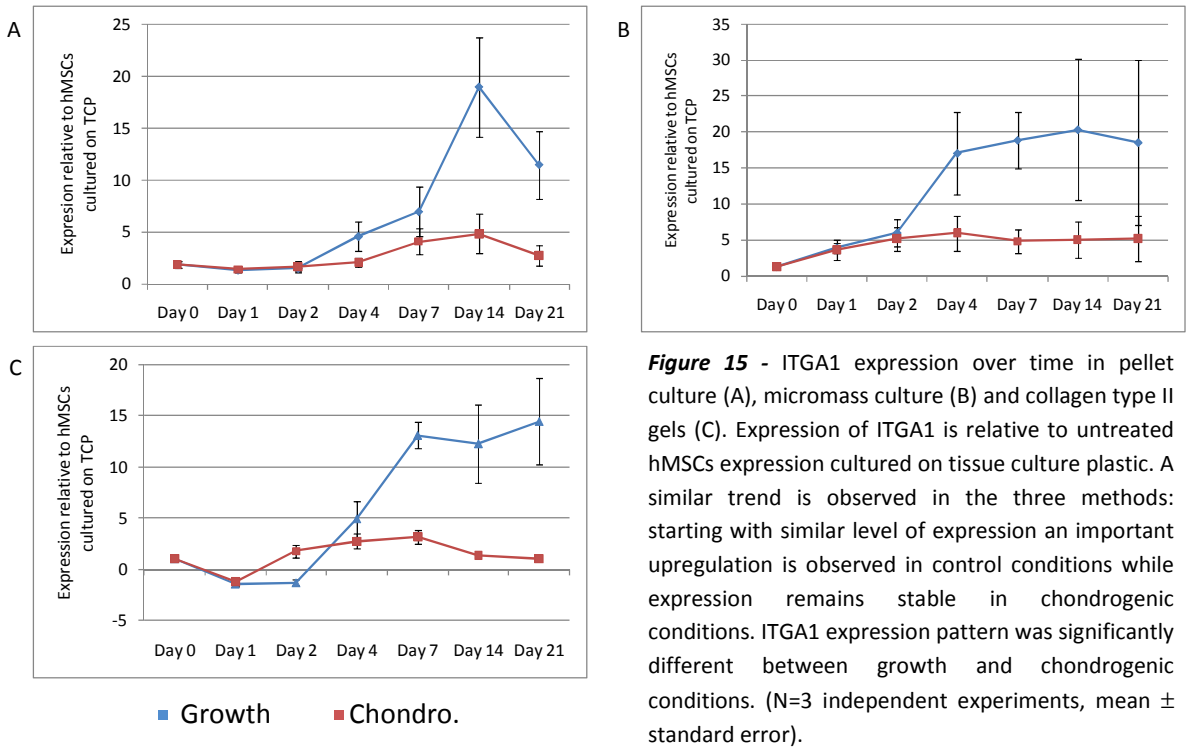


Figure 15 **-** ITGA1 expression over time in pellet culture (A), micromass culture (B) and collagen type II gels (C). Expression of ITGA1 is relative to untreated hMSCs expression cultured on tissue culture plastic. A similar trend is observed in the three methods: starting with similar level of expression an important upregulation is observed in control conditions while expression remains stable in chondrogenic conditions. ITGA1 expression pattern was significantly different between growth and chondrogenic conditions. (N=3 independent experiments, mean ± standard error).

ITGA2:

Here again the same trend is observed in the three different culture methods. Indeed, starting with similar initial levels of ITGA2 expression in growth and chondrogenic conditions, the expression of ITGA2 was importantly downregulated in the latter after Day 7 in each culture methods while remained relatively constant in the control samples (***Figure 16***). The differences in expression level between growth and chondrogenic conditions were significant in pellet culture and in collagen type II gels but not in micromass culture. Overall the expression of ITGA2 was higher in cells cultured in growth medium than in cells undergoing chondrogenic differentiation.

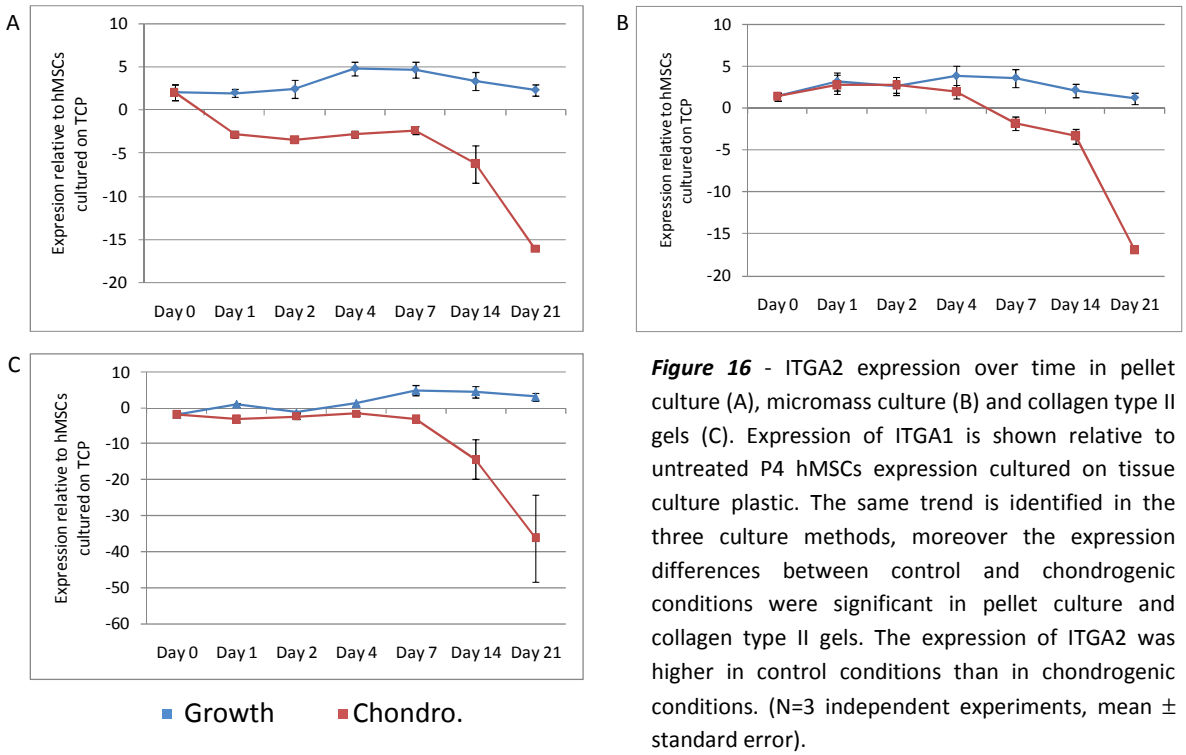


Figure 16 - ITGA2 expression over time in pellet culture (A), micromass culture (B) and collagen type II gels (C). Expression of ITGA1 is shown relative to untreated P4 hMSCs expression cultured on tissue culture plastic. The same trend is identified in the three culture methods, moreover the expression differences between control and chondrogenic conditions were significant in pellet culture and collagen type II gels. The expression of ITGA2 was higher in control conditions than in chondrogenic conditions. (N=3 independent experiments, mean ± standard error).

ITGA3:

The variation of ITGA3 expression showed analogous behaviour in all three culture methods (***Figure 17***). At early stages of chondrogenesis, ITGA3 expression is almost identical in control and chondrogenic groups, but at later time points the expression in hMSCs grown in chondrogenic medium tends to decrease, while expression in control cells remains at constant level, an expression similar to untreated hMSCs. The expression differences between growth and chondrogenic conditions were significant from Day 2 onwards in pellet culture, while for collagen II 3D hydrogels significance was reached from Day 7 onwards and in micromass culture only Day 14 showed significant difference between the two conditions. Moreover the expression profile was significantly different over time between the growth and chondrogenic conditions in pellet culture and in collagen gels. Most interestingly, on Day 21 the ITGA3 expression in growth condition in each method reached the same expression in hMSC cultured on TCP in collagen type II gels. The expression was importantly downregulated at the first stage but was upregulated from Day 2 onwards to reach a plateau around -1. In micromass culture, the expression of ITGA3 on Day 0 was five times less than in untreated hMSCs and this value is similar to those found in pellet culture and collagen type II gels on Day 2, this similarity might be explained by the protocol to forme micromass. Cells are thus cultured at high density before what was stated as Day 0 in this experiment and might explained the shift we observed when expression in micromass is compared to expression in pellet culture and collagen type II gels. Here again the expression of ITGA3 was overall higher in growth conditions compared to expression in cells cultured in chondrogenic medium.

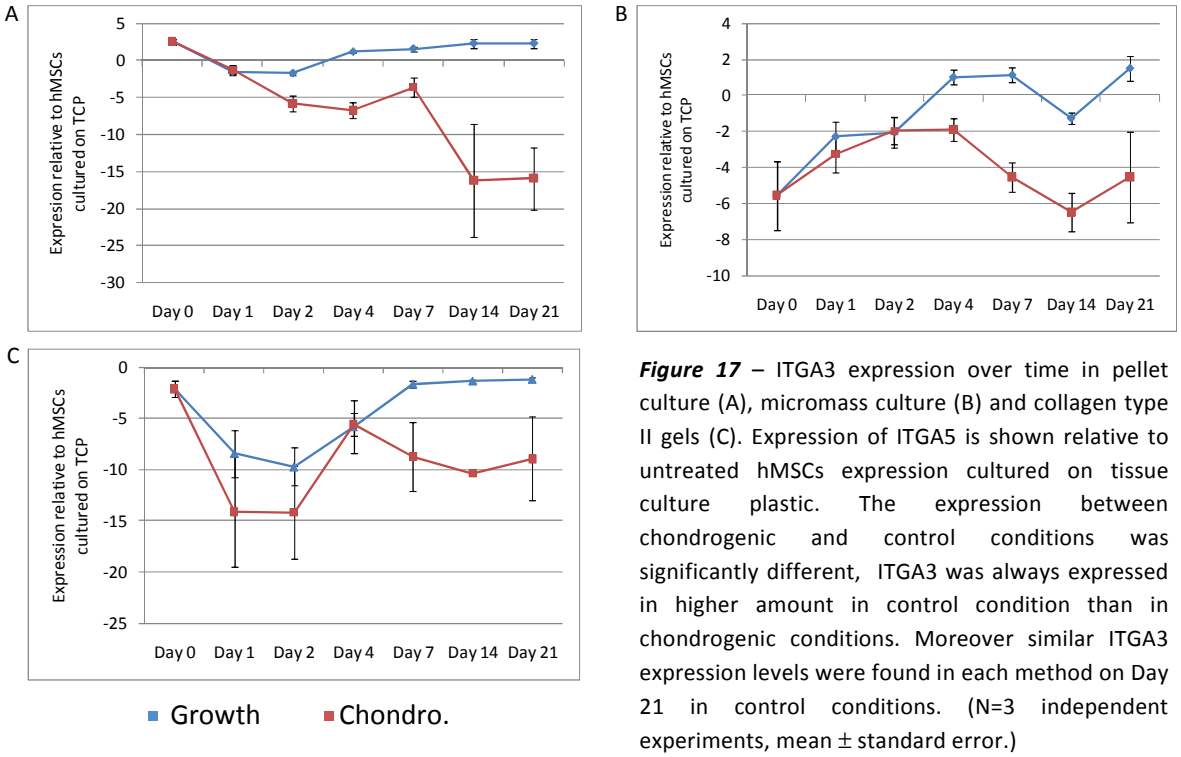


Figure 17 – ITGA3 expression over time in pellet culture (A), micromass culture (B) and collagen type II gels (C). Expression of ITGA5 is shown relative to untreated hMSCs expression cultured on tissue culture plastic. The expression between chondrogenic and control conditions was significantly different, ITGA3 was always expressed in higher amount in control condition than in chondrogenic conditions. Moreover similar ITGA3 expression levels were found in each method on Day 21 in control conditions. (N=3 independent experiments, mean ± standard error.)

ITGA4:

Throughout the 21 days, ITGA4 always more expressed in hMSC cultured in growth medium than hMSC undergoing chondrogenesis in all three different culture methods (***Figure 18***). Furthermore, the expression variations followed similar patterns between growth and chondrogenic conditions. However from Day 4 to Day 21 in micromass culture and collagen II gels there is noticeable difference in expression between these conditions, ITGA4 being importantly less expressed in chondrogenic conditions compared to growth conditions. These differences were statistically significant in collagen II 3D gels, but not in micromass due to high variability between samples within the same day. Yet the same trend is graphically observed. Both in pellet culture and collagen II gels the expression changes throughout the differentiation can be divided into two steps: an initial downregulation in both conditions from an expression initially similar or double the expression found in untreated hMSCs to values up to 8 times lower than in untreated hMSCs on Day2, the second steps is the upregulation of ITGA4 from Day 2 until the end of experiment. While in pellet culture and collagen type II gels, ITGA4 expression was similar to untreated hMSCs on Day 0, in micromass culture the expression was found 4 times lower, which are values found on Day 2 in the two other culture models. Interestingly the final expression levels were similar in growth condition throughout the three different methods with values oscillating between -1.7 and +1-time fold expression in untreated hMSCs and between -2.7 and -5.85 in chondrogenic medium. Finally overall expression was found higher in cells cultured in growth medium than in cells undergoing chondrogenesis.

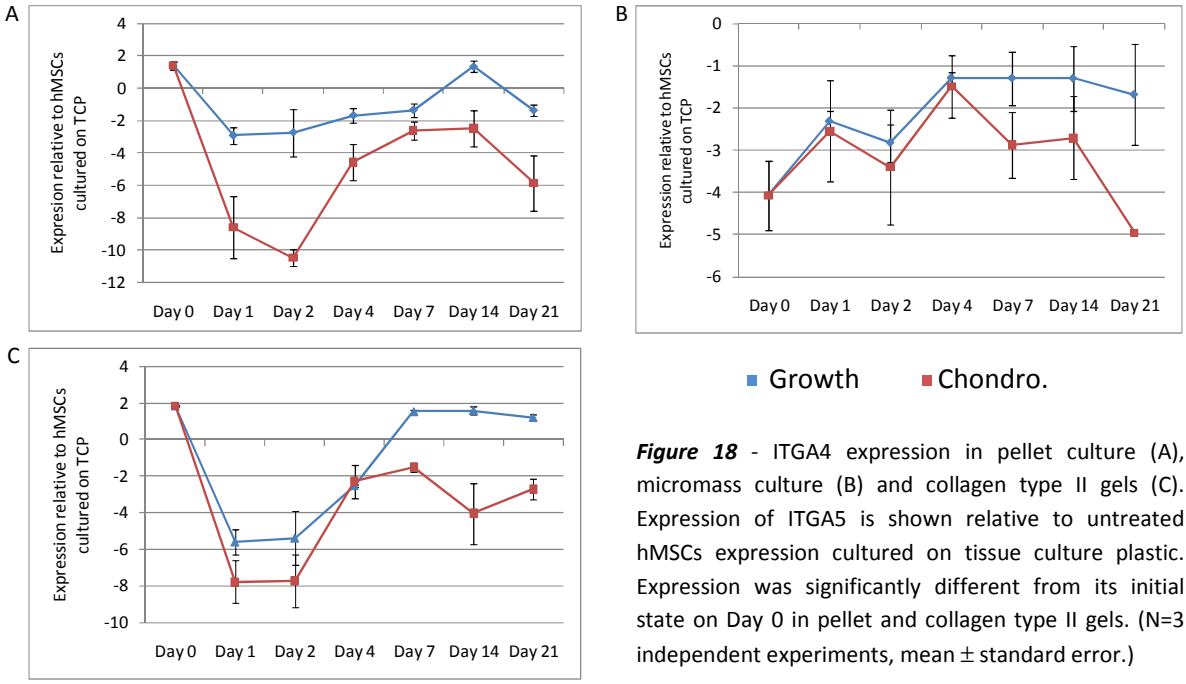


Figure 18 - ITGA4 expression in pellet culture (A), micromass culture (B) and collagen type II gels (C). Expression of ITGA5 is shown relative to untreated hMSCs expression cultured on tissue culture plastic. Expression was significantly different from its initial state on Day 0 in pellet and collagen type II gels. (N=3 independent experiments, mean ± standard error.)

ITGA5:

ITGA5 expression in growth conditions remained stable in the three methods, indeed its level oscillate at similar expression levels found in untreated hMSCs cultured on TCP (***Figure 19***). While in chondrogenic conditions the expression variations were different depending on the method:

- In pellet culture, ITGA5 was continuously downregulated throughout the differentiation process.
- In micromass culture ITGA5 was initially slightly upregulated followed by a downregulation after Day 4 to eventually reach the same expression level than in growth conditions. Yet these variations are little and not significant.
- In collagen II gels, expression in cells undergoing chondrogenesis the remained stable until Day 4 from which it started to be greatly downregulated, on Day 14 and 21 expression was 12 time lower than in untreated hMSCs.

Interestingly, the expression of ITGA5 on Day 0 of micromass culture was 2-time lower than expression in untreated hMSCs and this value correspond to expression level found both in pellet culture and collagen type II gels on Day 2, this might be due to the 48 hours time shift. Moreover in pellet culture and collagen gels the expression of ITGA5 in chondrogenic conditions was found much lower than the expression in growth conditions. The expression pattern was significantly different over time between the growth and chondrogenic conditions in micromass culture and in collagen gels.

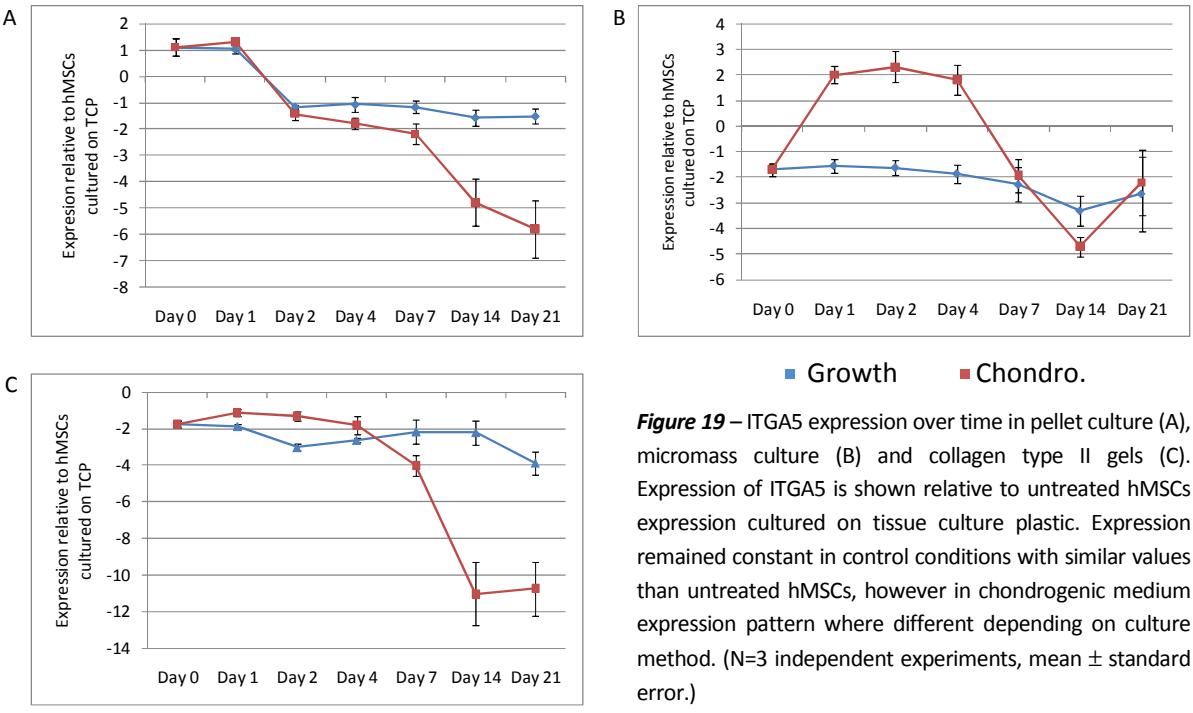


Figure 19 – ITGA5 expression over time in pellet culture (A), micromass culture (B) and collagen type II gels (C). Expression of ITGA5 is shown relative to untreated hMSCs expression cultured on tissue culture plastic. Expression remained constant in control conditions with similar values than untreated hMSCs, however in chondrogenic medium expression pattern where different depending on culture method. (N=3 independent experiments, mean ± standard error.)

ITGA6:

ITGA6 showed dissimilar expression pattern according to the culture methods. In pellet culture, ITGA6 expression was firstly slightly downregulated in growth conditions then reached a plateau on Day 1, while in chondrogenic medium expression was importantly downregulated, moreover the differences among the two conditions were significant (***Figure 20***). On the other hand, in micromass, ITGA6 expression in growth and in chondrogenic condition varied in correspondingly characterised by a continuous increase, and the expression levels were similar at each time point. Interestingly, the level of expression of ITGA6 found on Day 0 in micromass culture, approximately 15-times less expressed than in untreated hMSCs, was similar to the expression found on Day 2 in the two other culture models, this is explained by the 48 hours shift for micromass formation by gravity. In collagen type II gels, the high variability were found between the samples along with missing data on Day 14 and Day 21 in chondrogenic conditions does not allow any conclusion. Finally, here again in growth conditions the expression levels in all three methods converged toward a similar values on Day 21 which is about -3 times expression in untreated hMSCs, this level of expression was reach from Day 1 in pellet culture, from Day 2 in micromass culture and from Day 7 in collagen gels.

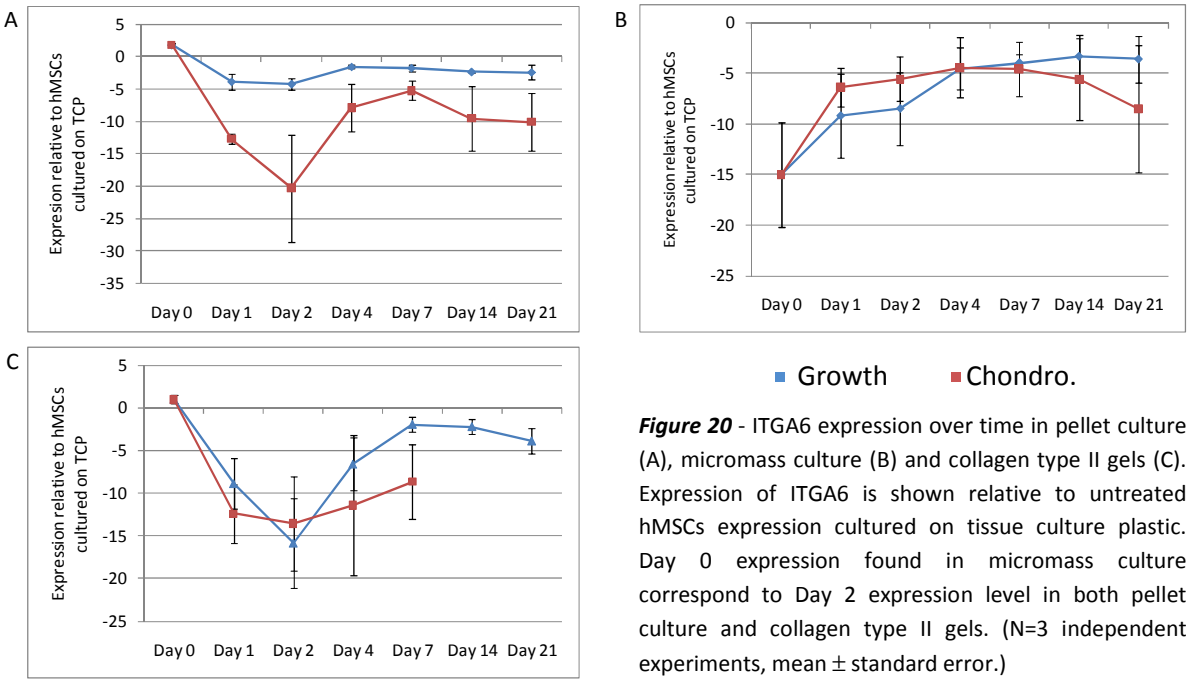


Figure 20 - ITGA6 expression over time in pellet culture (A), micromass culture (B) and collagen type II gels (C). Expression of ITGA6 is shown relative to untreated hMSCs expression cultured on tissue culture plastic. Day 0 expression found in micromass culture correspond to Day 2 expression level in both pellet culture and collagen type II gels. (N=3 independent experiments, mean ± standard error.)

ITGA7:

No clear expression pattern could be identified for ITGA7 between the different culture models (***Figure 21***). However in pellet culture and in micromass culture, expression in growth and chondrogenic conditions varied identically. In pellet culture, an initial downregulation from Day 0 to Day 2 followed by an upregulation until Day 14 and a final decrease on Day 21. No significant differences are seen between growth and chondrogenic conditions, and no significant expression changes were noticed compared to initial state Day 0. Similarly, in micromass culture no differences were observed between the growth and chondrogenic conditions, moreover expression in control and in chondrogenic conditions varied in correspondingly. It seems that ITGA7 expression is not affected at all by the chondrogenic differentiation. No significant conclusion was possible, neither in collagen II gels were the last two time points are missing for hMSC undergoing chondrogenesis. The expression pattern was not significantly different over time between the growth and chondrogenic conditions in any culture system.

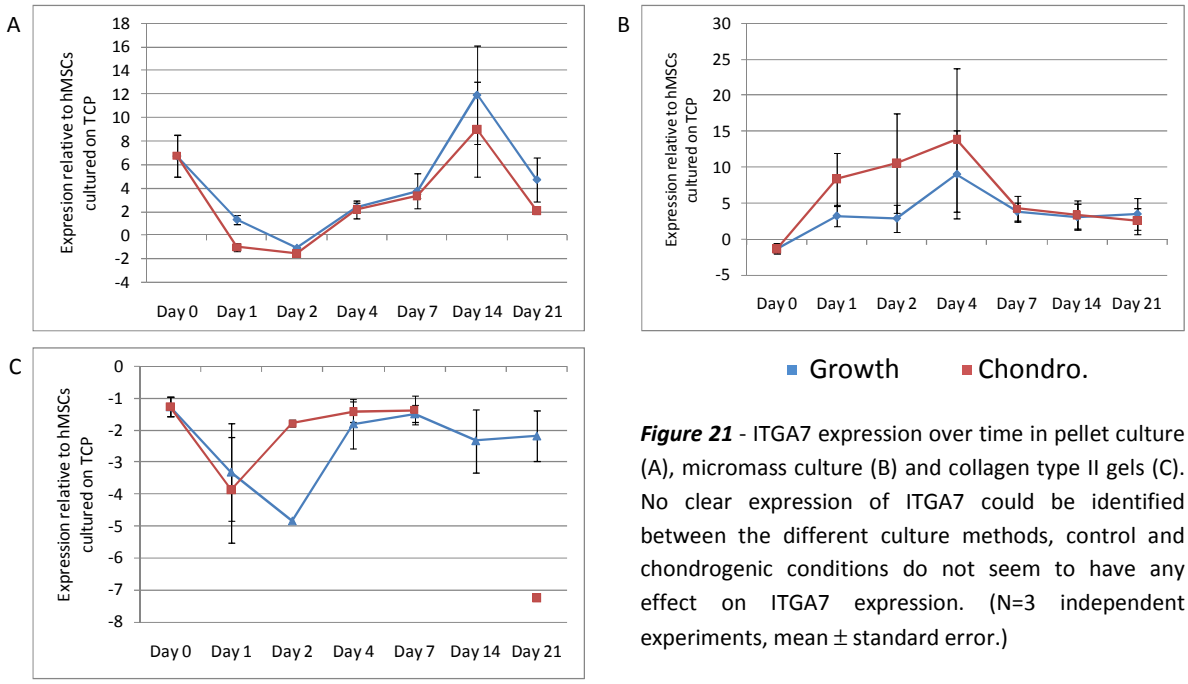


Figure 21 - ITGA7 expression over time in pellet culture (A), micromass culture (B) and collagen type II gels (C). No clear expression of ITGA7 could be identified between the different culture methods, control and chondrogenic conditions do not seem to have any effect on ITGA7 expression. (N=3 independent experiments, mean ± standard error.)

ITGA10:

ITGA10 had a similar expression pattern between pellet culture and collagen type II gels, indeed in both methods the expression in growth conditions was significantly downregulated compared to Day 0 at early stages followed by an important upregulation after Day 4 (***Figure 22***). Moreover in chondrogenic conditions the expression remained relatively unchanged. In micromass culture, both growth and chondrogenic the same expression pattern was identified characterised by an initial upregulation at similar rate followed by a downregulation which was greater in chondrogenic conditions resulting in an overall higher expression in growth conditions, however the variability was particularly important in micromass and did not lead to any conclusive results. It is interesting to notice that Day 0 expression level in micromass culture is similar to Day 2 expression level in pellet and micromass culture, here again the expression pattern of micromass might be shifted by 48 hours due to the process of micromass formation. In the three different methods the expression was higher in growth than in chondrogenic conditions. The expression profile over time was significantly different between the growth and chondrogenic conditions in pellet culture and in collagen gels, but not in micromass culture due to a high variability.

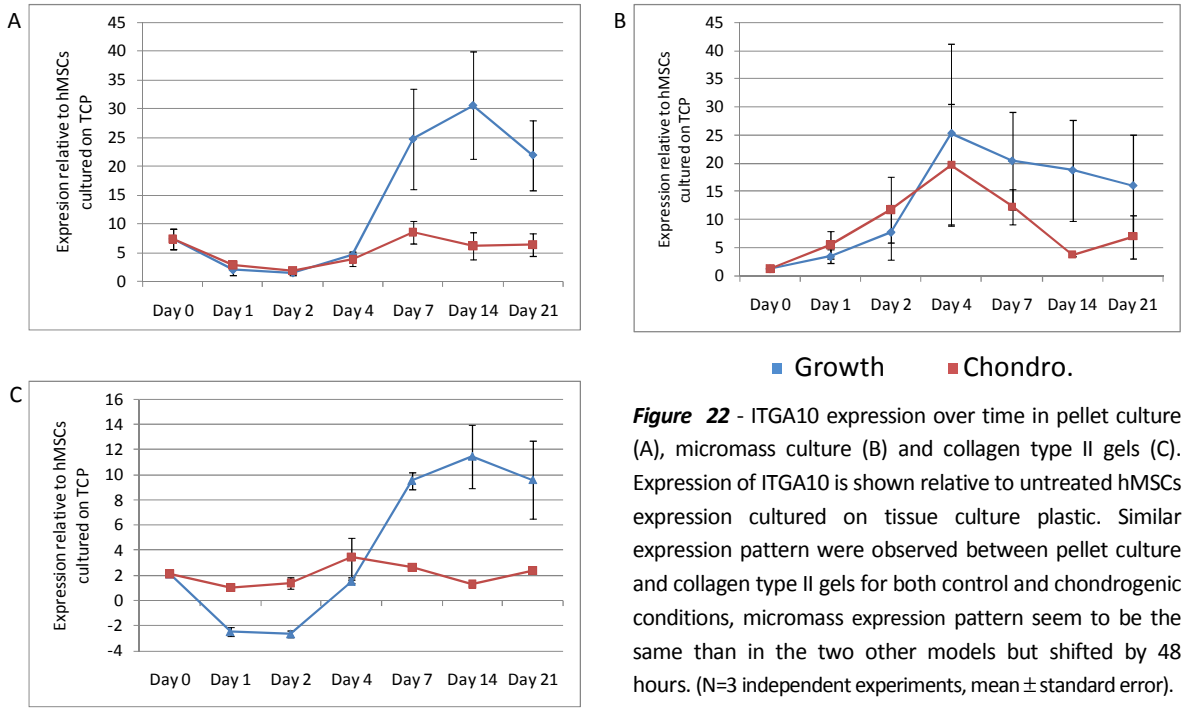


Figure 22 - ITGA10 expression over time in pellet culture (A), micromass culture (B) and collagen type II gels (C). Expression of ITGA10 is shown relative to untreated hMSCs expression cultured on tissue culture plastic. Similar expression pattern were observed between pellet culture and collagen type II gels for both control and chondrogenic conditions, micromass expression pattern seem to be the same than in the two other models but shifted by 48 hours. (N=3 independent experiments, mean ± standard error).

ITGA11:

ITGA11 was one of the two alpha subunits where expression was found higher in chondrogenic conditions than in growth conditions, and even if this difference was not significant this trend was however found in the three culture models (***Figure 23***). Indeed the expression profile over time was not significantly different between the growth and chondrogenic conditions in any of the culture systems. Moreover the expression pattern of ITGA11 in hMSCs in growth conditions and those undergoing chondrogenesis was similar, and a significant gradual upregulation was observed in the three models for both conditions. In pellet culture, stable expression level were observed until Day 7 were an important increase in expression occurred in both condition. In micromass culture, the expression was continuously and greatly upregulated. Overall the expression pattern was similar between growth and chondrogenic conditions with a slightly higher expression in chondrogenic conditions. Finally, in collagen gels the expression did not change importantly however a continuous increase was noticed.

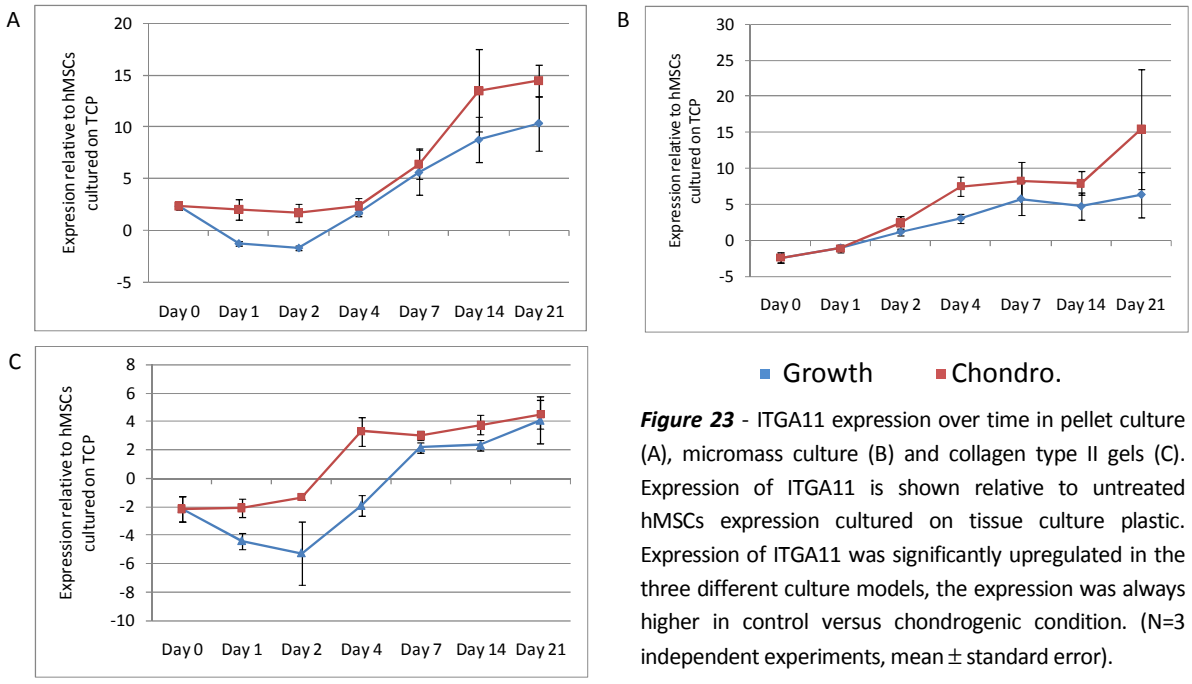


Figure 23 - ITGA11 expression over time in pellet culture (A), micromass culture (B) and collagen type II gels (C). Expression of ITGA11 is shown relative to untreated hMSCs expression cultured on tissue culture plastic. Expression of ITGA11 was significantly upregulated in the three different culture models, the expression was always higher in control versus chondrogenic condition. (N=3 independent experiments, mean ± standard error).

ITGAV:

ITGA11 was the second alpha subunit where expression was found to be higher in chondrogenic conditions over growth conditions in all three methods. Yet the expression pattern was different according to the culture methods (***Figure 24***). In pellet culture, the variations in expression levels remained small, characterised by an initially downregulation followed by a significant upregulation after Day 2 in both growth and chondrogenic conditions. In micromass, expression was significantly increased in a continuous manner in both conditions however the increase was more important in hMSCs undergoing chondrogenesis. In collagen type II gels, ITGAV expression remained stable in chondrogenic conditions while in growth conditions it was significantly downregulated at early time points, followed by an upregulation to reach the same expression level than in chondrogenic conditions on Day 21. Importantly in the three culture models, the expression level of ITGA11 in growth and chondrogenic conditions converge to a similar level on Day 21. Furthermore the ITGA11 expression was overall higher in hMSCs undergoing chondrogenesis than hMSCs cultured in growth medium.

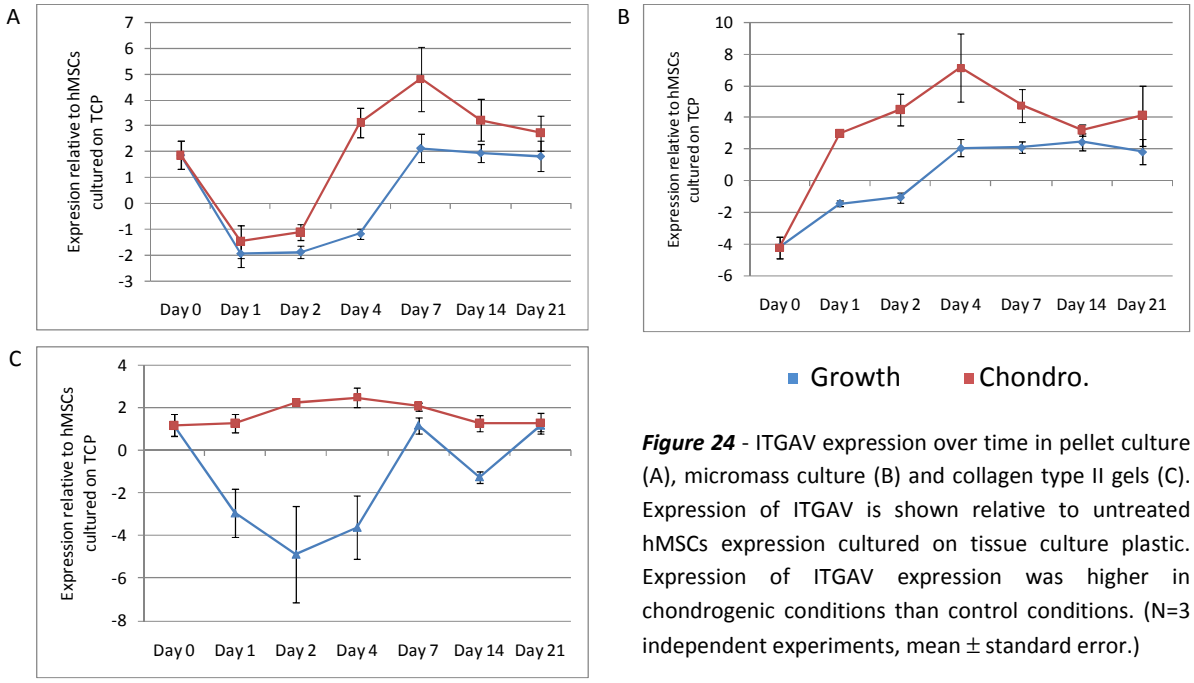


Figure 24 - ITGAV expression over time in pellet culture (A), micromass culture (B) and collagen type II gels (C). Expression of ITGAV is shown relative to untreated hMSCs expression cultured on tissue culture plastic. Expression of ITGAV expression was higher in chondrogenic conditions than control conditions. (N=3 independent experiments, mean ± standard error.)

4.2.5. Expression of Beta Integrin Subunits in the Different Culture Systems

ITGB1:

The expression of the β1 subunit was very similar between growth and chondrogenic conditions (***Figure 25***). In pellet culture and collagen type II gels, the expression pattern of ITGB1 can be divided in two steps: the first step is characterized by a downregulation from Day 0 to Day 1 at similar rate between the growth and chondrogenic conditions in both culture models. The second step occurs from Day 2 until the end of the experiment and is chacterized by an upregulation in the two conditions.

In micromass culture the expression seems to be only upregulated, yet probably that first step of the expression pattern which is the downregulation occurred during the 48 hours necessary for the spontaneous formation of the micromass and thus is seen, only the second step of the pattern is identified from Day 2 which is the upregulation of ITGB1 expression.

The expression profile of ITGB1 was not significantly different over time between the growth and chondrogenic conditions in micromass and pellet culture, but was different in collagen gels.

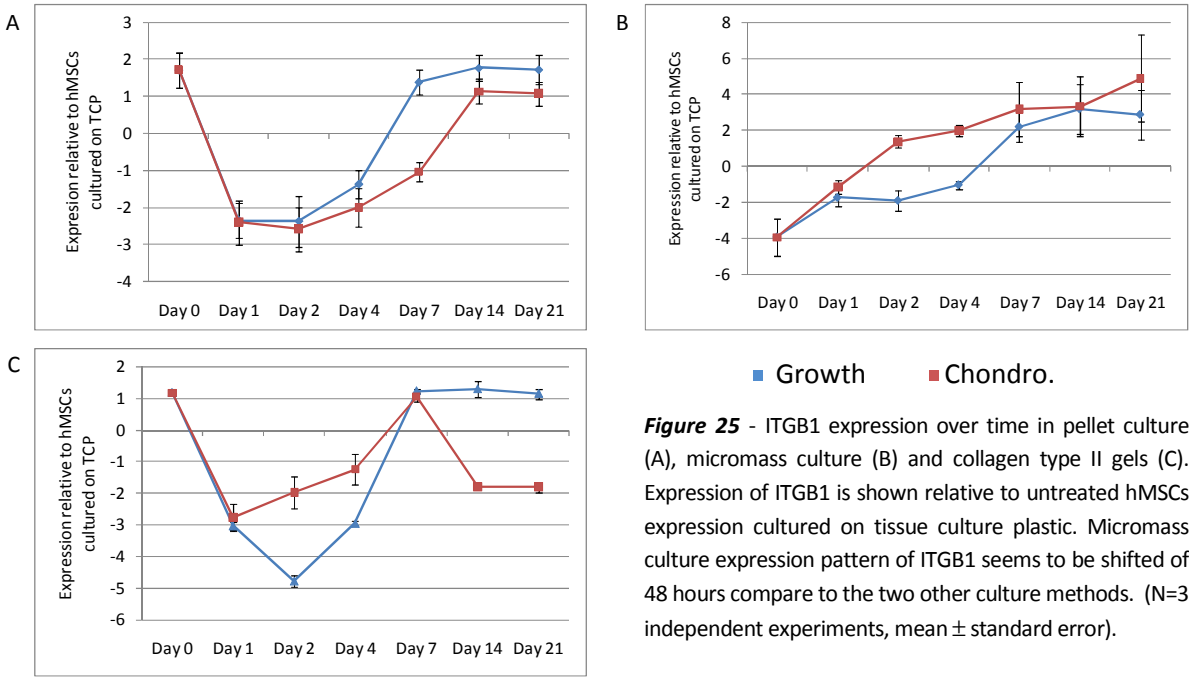


Figure 25 - ITGB1 expression over time in pellet culture (A), micromass culture (B) and collagen type II gels (C). Expression of ITGB1 is shown relative to untreated hMSCs expression cultured on tissue culture plastic. Micromass culture expression pattern of ITGB1 seems to be shifted of 48 hours compare to the two other culture methods. (N=3 independent experiments, mean ± standard error).

ITGB3 :

Data are missing for the latest time points for the three methods, yet an important downregulation is observed on Day 14 in hMSCs undergoing chondrogenesis and this is true for the three culture models (***Figure 26***). Cells cultured in growth conditions had a constant expression of ITGB3, while in chondrogenic conditions ITGB3 expression was downregulated, however these differences in expression level were not statistically different, this might be due to some data points that are lacking. The expression pattern of ITGB3 over time was not significantly different between the growth and chondrogenic conditions in any of the culture systems. Overall expression of ITGB3 was higher in growth conditions compared to chondrogenic conditions.

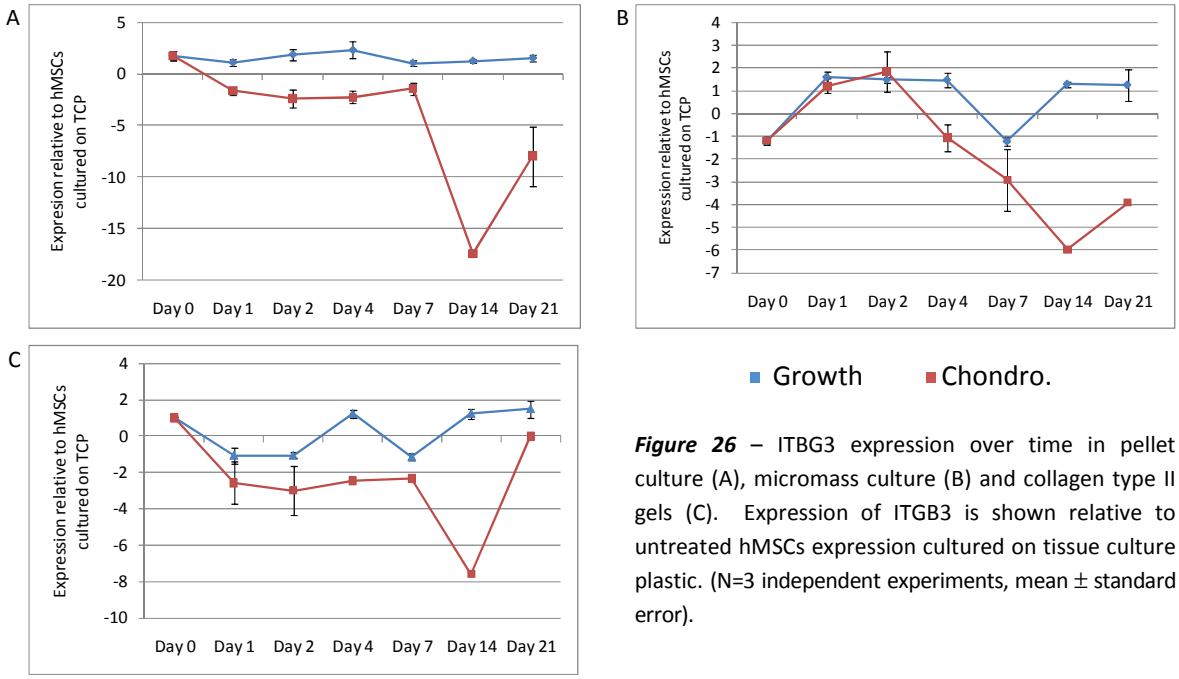


Figure 26 – ITBG3 expression over time in pellet culture (A), micromass culture (B) and collagen type II gels (C). Expression of ITGB3 is shown relative to untreated hMSCs expression cultured on tissue culture plastic. (N=3 independent experiments, mean ± standard error).

ITGB5:

The expression of ITGB5 follows the same pattern in pellet culture and collagen type II gels for both condition which are growth and chondrogenic medium, which is characterized by an initial downregulation from Day 0 to Day 1, followed by a plateau between Day 1 and Day 2, and an upregulation from Day 2 onwards (***Figure 27***). In the two culture models, the upregulation was more important in growth conditions than chondrogenic conditions. In micromass, the expression pattern of ITB5 follows the one seen in pellet culture and collagen II gels from Day 2: an upregulation in both conditions, however an higher increase in growth over chondrogenic conditions. Again this shift in time for the expression pattern might be linked to the 48 hours necessary for the formation of micromass. The expression profile of ITGB5 was significantly different over time between the growth and chondrogenic conditions in the three methods.

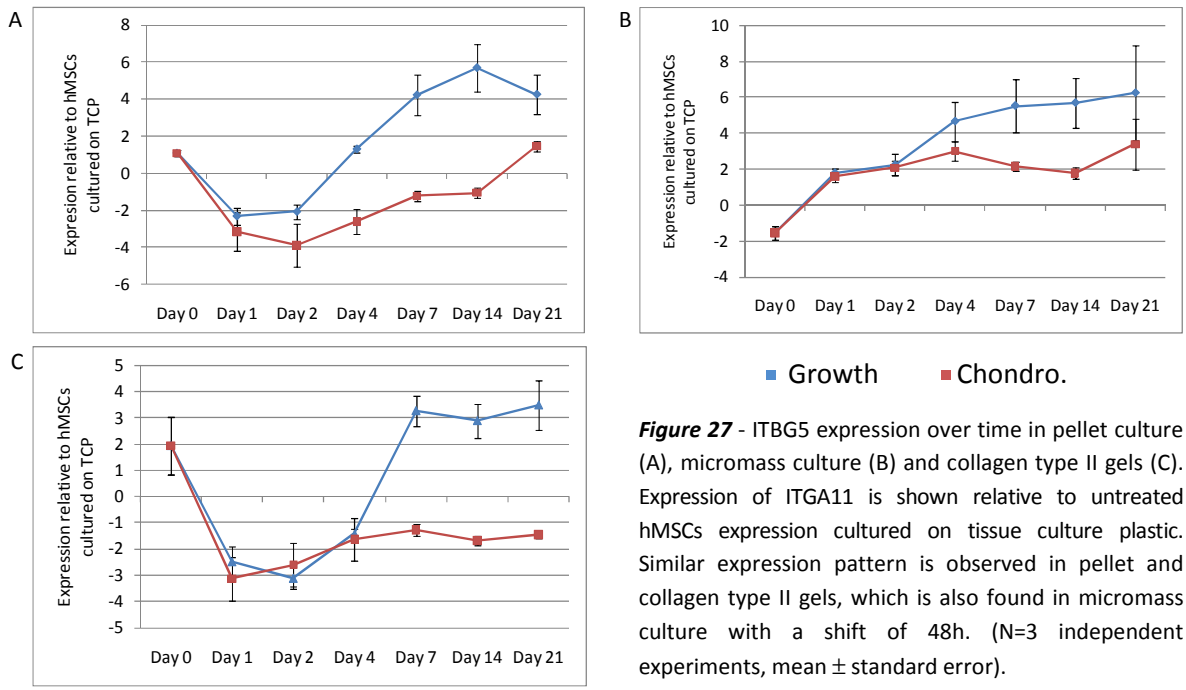


Figure 27 - ITBG5 expression over time in pellet culture (A), micromass culture (B) and collagen type II gels (C). Expression of ITGA11 is shown relative to untreated hMSCs expression cultured on tissue culture plastic. Similar expression pattern is observed in pellet and collagen type II gels, which is also found in micromass culture with a shift of 48h. (N=3 independent experiments, mean ± standard error).

ITGB8:

In the three different culture methods, the expression of ITGB8 was greatly increased in growth conditions while remained constant in chondrogenic conditions, except for collagen II gels where expression in hMSCs undergoing chondrogenesis was also continuously upregulated (***Figure 28***). Indeed in growth conditions, ITGB8 expression was increased up to 50 times expression level found in untreated hMSCs. The expression profile over time was significantly different between the growth and chondrogenic conditions in pellet culture and in micromass culture, but not in collagen type II gels due to the important variability of the expression of ITGB8 within the same group of samples. Overall expression of ITGB8 in growth conditions were much higher than in chondrogenic conditions.

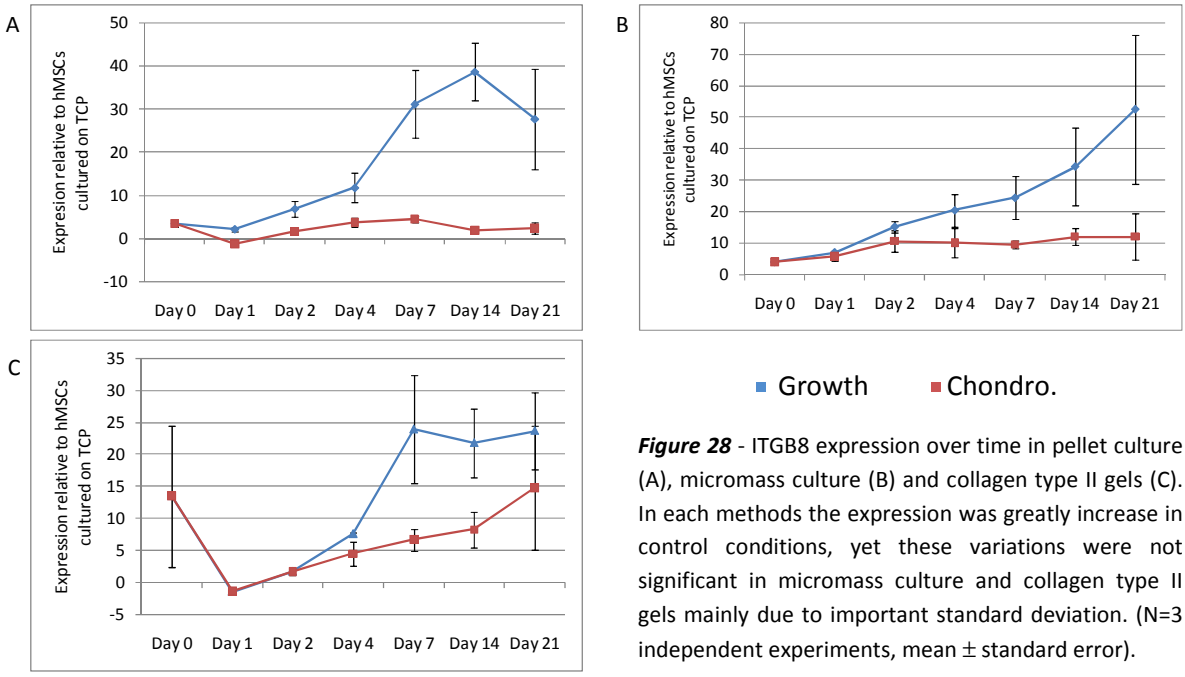


Figure 28 - ITGB8 expression over time in pellet culture (A), micromass culture (B) and collagen type II gels (C). In each methods the expression was greatly increase in control conditions, yet these variations were not significant in micromass culture and collagen type II gels mainly due to important standard deviation. (N=3 independent experiments, mean ± standard error).

5. Discussion

The aim of the project is to establish a transcript expression profile for each integrin subunits in different culture systems during hMSC chondrogenesis. Cartilage tissue engineering struggles to produce stable chondrocyte and integrins are major intermediates between cells and the ECM, establishing their expression pattern throughout chondrogenic differentiation would provide an improved knowledge about the adhesion requirements but also about the integrin-triggered intracellular signalling required for the differentiating cells.

5.1. Statistical Significance

In this study, we performed two tailed *t*-test between the growth and chondrogenic conditions for each method on each day. We also performed two-way ANOVA in order to determine if the transcript expression between the growth and chondrogenic conditions was significantly different over time. However although graphically the behaviour between these two conditions was clearly distinct, statistical significance was not always established. This was mainly due to variability in expression within the same day between the three independent experiments for each culture methods in either growth or chondrogenic conditions. In the present study, we were more interested in showing trends in expression pattern of the different integrins subunits between different culture models.

5.2. Chondrogenesis: Comparison Between Different Culture Systems

We first wanted to determine the relative success of chondrogenesis in our three culture systems. The qPCR data and histology staining revealed that the overall collagen deposition and mRNA expression, which includes collagen types I, II and X, was greater in pellet culture than in the other culture methods. Collagen staining by Sirius red was weaker in micromass culture compare to pellet culture and finally the collagen II hydrogels showed the lowest overall collagen expression. Moreover, pellet culture showed also the higher *COL2A1* expression reaching up to 1.4-times *GAPDH* expression level on Day 21. In collagen gels, expression reached 0.8-time GAPDH expression but was only of 0.27-time *GAPDH* expression in micromass culture. However *COL2A1* expression was detected earlier in collagen gels than in pellet culture. *COL1A1*, characteristic of fibrocartilage, was 16 and 11-times higher *COL2A1* in pellet culture and collagen II gels on Day 21. In micromass culture, *COL1A1* was over 100 times more expressed than *COL2A1*. For the third type of collagen quantified, *COL10A1*, pellet expression reached 15-times *GAPDH* expression on Day 21, compare to only 5.5.times and 2-times *GAPDH* expression in collagen gels and micromass respectively. In conclusion, micromass culture showed the higher *COL1A1* expression and the lower *COL2A1*, the collagen RNA being produced suggests that the cell may be producing an ECM more characteristic of fibrocartilage than hyaline cartilage. On the other hand, pellet culture showed the greatest overall collagen deposition and *COL2A1* expression (***Table 8***), however it also had the higher *COL10A1* suggesting that chondrocytes might eventually undergo hypertrophy. Finally in collagen type II gels, the collagen deposition was the lowest when looking at collagens expression, yet relatively to *COL2A1* it expressed less *COL1A1* and *COL10A1* than in pellet and micromass culture.

Table 8 - Collagen types I and X relative expression to Collagen type II expression

	Pellet culture	Micromass culture	Collagen type II gels
$\frac{COL1A1}{COL2A1}$ *expression*	16	115	11
$\frac{COL10A1}{COL2A1}$ *expression*	11	7	7

Concerning the chondrocyte phenotype markers *ACAN* and *SOX9*, no significant differences were noticed between cells grown in growth or chondrogenic medium. *SOX9* is an early chondrogenic transcription factor which has been shown to activate *COL2A1* and *ACAN* expression, its expression was slightly upregulated in all the methods in both conditions and this weak upregulation seems to be sufficient to support matrix production. In micromass culture, *ACAN* expression tended to increase over time, while in the two other culture models its expression was downregulated. Although investigating chondrocyte phenotype markers expression investigation by qPCR did not show upregulation as expected during chondrogenesis, histology confirmed accumulation of proteoglycans in the extracellular environment by a bright alcian blue staining. Finally it is also important to highlight the fact that *PPARγ* was never expressed, and that *RUNX2* expression levels remained very low. The hMSCs are thus not committed to either adipogenic or osteogenic fates; some rare cells might undergo osteogenesis as shown by the low level of *RUNX2* however it would be a negligible fraction.

5.3. Time-shift in Integrin Transcript Expression Pofile in Micromass Culture

The formation of micromass involves incubating the cells in growth medium in a round bottom well of a 96-well plate such that the micromass spontaneously forms within 48 hours by falling due to gravity cells then reinforce the interactions by forming cell-cell contacts, Day 0 was defined as when medium was changed for chondrogenic medium, hence cells are exposed to a high cellular density system which promotes chondrogenic differentiation 48 hours prior the experimental official Day 0. This might result in a time-shift of 24-48 hours in integrin expression in micromass culture versus pellet culture and collagen type II 3D hydrogels. Indeed for several integrin subunits, a similar expression pattern was not identifiable between the three culture methods due to this time-shift, for some integrins subunits a 24 to 48 hours shift should be taken into consideration in order to recognise an expression pattern between the models.

We are fairly confident that a time shift is being observed in expression of the integrin subunits α3, α4, α6 and β1. A downregulation of integrin subunits α3, α4 and α6 is seen from Day 0 to Day 2 which is followed by an upregulation from Day2 onwards in both pellet culture and in collagen type II gels, however the initial downregulation is not seen in micromass culture probably due to the 48 hours time-shift. Furthermore the expression pattern of α3, α4, α6 and β1 in micromass follows the expression pattern in the two other culture models after Day 2. Concerning integrin subunit α5 and α10 expression level on Day 0 in micromass might correspond to Day 2 in pellet culture and collagen type II gels however after Day 2 the expression pattern did not follow, hence it is not possible to affirm if a time-shift occurred in the expression of these two integrin subunits.

5.4. Integrin Subunits Expression

The most interesting approach to analyse the expression profile of integrins during chondrogenesis is to look at integrin subunits transcript expression evolution according to their ligand affinity. Hence we will discuss about the integrin expression profile variations for the alpha subunits related to the collagen-, fibronectin- and laminin-binding integrins separately.

5.4.1. Integrins Receptors for Collagen

Collagen constitutes one of the major component of cartilage. The four primarily collagen-binding integrins are $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$ and $\alpha11\beta1$, they are known to be widely expressed on chondrocytes. As the alpha subunits $\alpha1$, $\alpha2$, $\alpha10$ and $\alpha11$ are only associated with $\beta1$ and not any other beta subunit, the alpha subunits expression is directly representative to the corresponding integrin expression. Furthermore the expression pattern of integrin and the ligand affinity of the integrin defines the adhesive property of the cell.

The integrin subunit $\alpha1$ expression was stable in chondrogenic medium while greatly upregulated in growth conditions in all three culture methods compared to untreated hMSCs cultured on tissue culture plastic. Conversely, the expression of integrin subunit $\alpha2$ was stable in growth conditions but importantly downregulated in hMSCs undergoing chondrogenic differentiation. Similar to the integrin subunit $\alpha1$, $\alpha10$ was also highly upregulated in growth conditions, and remained stable during chondrogenesis in pellet culture and collagen type II gels while also upregulated in micromass. Finally, the integrin subunit $\alpha11$ expression was upregulated in both conditions, yet more importantly in chondrogenic conditions resulting in an overall higher expression in hMSCs undergoing chondrogenesis. With the exception of $\alpha11$, the three other alpha subunits expression was higher in growth conditions over chondrogenic conditions. Taken together these data suggest that cells undergoing chondrogenesis are less adhesive to collagen than cells grown in growth medium. The integrin $\alpha1\beta1$ is a primary chondrocyte collagen type VI receptor and collagen type IV, however it also binds to a lower extent to collagen types I, II.[74] The integrin $\alpha2\beta1$ binds preferentially collagen type I, yet it is also a receptor for collagen type IV, VI. $\alpha10\beta1$ integrin binds mostly collagen type IV, then type VI and type II with a lower affinity. The last collagen-binding integrin $\alpha11\beta1$ is primary receptor for collagen type I, but it also binds to type IV. Finally, these four integrins are also receptor for collagen type IX.[56] From the data obtained in the present study, the cells culture in growth conditions in the three methods increased over time their overall adhesive properties to collagens, ***Table 9*** summarises the change in adhesion properties of hMSCs cultured in growth medium.

Table 9 - Adhesion properties of hMSCs cultured in growth medium. The overall adhesion to collagen type I, II, IV, VI and IX was enhanced over the time period of 21 days as the integrin α subunits related to collagen receptors are all upregulated.

Collagen type	Adhesion to collagen	Justification
Collagen type VI	Increased	Upregulation of $\alpha1$ and $\alpha11$
Collagen type IV	Increased	Upregulation of $\alpha1$, $\alpha10$ and $\alpha11$
Collagen type IX	Increased	Upregulation of $\alpha1$, $\alpha10$ and $\alpha11$
Collagen type II	Increased	Upregulation of $\alpha1$
Collagen type I	Increased	Upregulation of $\alpha1$, Unchanged expression of $\alpha2$

It is more difficult to derive any conclusion for hMSCs undergoing chondrogenesis about adhesion variations over time as the alpha subunit of both primary collagen I binding integrins, α1β1 and α11β1, were regulated in an exact opposite manner: α2 was downregulated while α11 was upregulated. Expression of integrin subunits α1 and α10 remained unchanged. Hence no conclusion is possible about adhesion to collagen type I, IV and IX, however it suggests that adhesive property remained unchanged. ***Table 10*** summarises the main change in adhesion requirement in hMSCs undergoing chondrogenesis.

Table 10 **-** Adhesion properties of hMSCs cultured in chondrogenic medium. No statement was possible about change in adhesion properties for collagen types I, IV and IX, adhesion to collagen type II remains unchanged as expression of both integrin subunits α1 and α10 while adhesion to collagen type VI decrease due a downregulation of integrin subunit α2.

Collagen type	Adhesion to collagen	Justification
Collagen type II	unchanged	α1 and α10 remained unchanged
Collagen type VI	decreased	downregulation of α2.

The expression of integrin receptors for collagen was increased over time in hMSCs culture in growth medium which implies an enhanced adhesion to these ECM molecules, while in hMSCs undergoing chondrogenesis overall adhesion over time remained stable due to unchanged integrin expression or counter regulated expression.

Varas *et al.* investigated the expression of integrins subunits α1, α10 and α11 and stated that during chondrogenesis in aggregate culture α1 and α11 are downregulated while α10 is upregulated.[63] These results are quite different from our data as α1 and α10 remained stable, and α11 was upregulated. However we observed an important increase of integrin subunit α10 expression in growth conditions. Loeser et al. showed that β1-integrins expression were upregulated in monolayer culture and alginate culture which in turn increase the adhesion properties of the cells, this upregulation was also observed in osteoarthritic cartilage compare to normal cartilage, and the greatest increase was observed for α1β1 integrin.[74] The important increase of integrin subunit α1 observed in our cell culture studies in growth conditions seems to be a feature of osteoarthritic cells, the fact that in chondrogenic condition the expression of α1 remained constant is encouraging. Furthermore, α2 expression was importantly downregulated in chondrogenic medium while remaining stable in growth conditions. This behaviour is not consistent with and Varas *et al.* study where α2 expression remained unchanged in cells undergoing chondrogenesis, yet in Goessler *et al.* study α2 expression was downregulated.[75] Moreover, integrin subunit α2 was not found significantly expressed in articular chondrocytes, and integrin α2β1 is known to more expressed in chondrosarcoma relative to normal adult chondrocytes.[74]

5.4.2. Integrins Receptors for Fibronectin, Vitronectin and Osteopontin

Fibronectin is a common extracellular matrix component in native cartilage tissue. Several integrins binds to fibronectin: integrins α4β1 and α4β7 recognise the LDV tripeptide found in fibronectin, while α5β1, αVβ1, αVβ3, αVβ6, αVβ5, αVβ8 recognise the RGD sequence. These integrins are not only receptor for fibronectin, they bind numerous other ligands among Osteopontin, COMP, vitronectin, tenascin etc. The beta subunits β6 and β7 were not expressed, hence we are only considering the following integrins: α4β1, α5β1, αVβ1, αVβ3, αVβ5, and αVβ8.

Interestingly the integrin subunits α4, α5, and αV present in the heterodimeric integrin receptors for fibronectin were the alpha subunit for which an expression pattern was not discernible between the three different culture methods we investigated. Indeed the expression of these three alpha subunits seemed to be highly variable and dependent on the culture model.

In pellet culture and collagen type II gels, α4 expression was initially greatly downregulated in chondrogenic conditions but was then increased again from Day 4, it seems that the fibronectin adhesion through the α4β1 integrin at early stages is diminished but then is increased again after Day 4. α4 is normally not expressed in normal adult articular chondrocytes, however in another study it's expression during chondrogenesis in pellet culture was tripled on D20 compared to D0.[74,75] In our cell culture study, α4 was overall downregulated in chondrogenic conditions, particularly on D1 and D2 in pellet culture and collagen type II gels before being slightly upregulated. Its expression was found relatively stable in growth conditions. α5β1 integrin is known to be the primary chondrocyte fibronectin receptor,[60] α5 expression remained stable in hMSCs undergoing chondrogenesis up to Day 7 in pellet culture and collagen type II gels, however on Day 14 and on Day 21 the expression of α5 was dramatically downregulated which implies a decreased adhesion to fibronectin via α5β1 integrin on late stages of chondrogenesis. This is consistent with another study where α5 subunit expression was also decreased however at a lower extent.[75] The expression pattern in chondrogenic environment in micromass was completely different, indeed the expression α5 was upregulated from D0 to D1. The plateau was maintained until Day 4 where the expression was decreased to a similar level than in growth conditions. Integrin α5β1 is known to be highly expressed on articular chondrocytes *in situ* and *in vitro*. Concerning the expression pattern of integrin subunit αV was here again depending on the culture method, however it is interesting to note that αV was more expressed in chondrogenic conditions than in growth conditions. Importantly, αV-containing integrins bind to vitronectin and osteopontin primarily and may serve as secondary fibronectin receptors.

Throughout the three culture models, α4 and α5 integrin subunits were more expressed in cells cultured in growth medium than in cells cultured in chondrogenic medium. Oppositely integrin subunit αV as found more expressed in cells culture in chondrogenic conditions. From these data, we can hypothesise that adhesion to fibronectin for cells cultured in growth medium occurs preferentially through α4β1 and α5β1 integrins, while for cells cultured in chondrogenic medium the adhesion to vitronectin and osteopontin via αV-containing integrins is more important. The αV-containing are αVβ1, αVβ3, αVβ5, and αVβ8, as β6 was not expressed.

5.4.3. Integrins Receptors for Laminin

The integrins α3β1, α6β1 and α7β1 are highly specific laminin receptors. α3β1 is also known to be a secondary receptor for fibronectin and collagen type II and α7β1 was recently proposed as a receptor for COMP.[76]

The expression α3 was dependant on the methods of culture, however overall we observed a downregulation throughout time in chondrogenic environment which is consistent with the findings of Varas *et al*. in aggregate culture.[63] At early stages, the expression of integrin subunit α3 was the same in growth or in chondrogenic conditions, however on Day 7, 14 and 21 an important downregulation was noticed in chondrogenic conditions compare to growth conditions, this was consistent in the three culture models. Hence the adhesive properties to laminin via the α3β1

integrin of the cells undergoing chondrogenesis seems to be decreased on late stage of differentiation compare to cells cultured in growth medium.

Concerning the integrin subunits α6, its expression pattern was highly variable depending upon the culture systems, indeed if its expression was downregulated in pellet culture it was found to be upregulated in micromass culture. Furthermore similar different behaviour was observed between the growth and chondrogenic conditions, because of this lack of divergence we believe that the integrin subunits α6 might not have a vital role in chondrogenesis. This hypothesis was also relevant for integrin subunit α7, indeed expression was identical between growth and chondrogenic conditions, whenever a variation occurred it was found in both conditions. Goessler *et al.* did not detect α6 nor α7 in their study during chondrogenesis and also concluded that the role of these integrin subunits was not important in chondrogenesis.[75]

5.4.4. Integrin Beta Subunits

The beta subunits β1, β3, β5, and β8 were expressed in hMSCs cultured in growth and in hMSCs cultured in chondrogenic medium. β2, β4, β6 and β7 were not detected.

The expression variations of beta subunits β1, β3, and β5 remained small which is consistent with other studies were expression of these subunits remained unchanged. Expression of β3 was downregulated on Day 14 and Day 21 in chondrogenic conditions, β3 is the beta component of two integrins αVβ3 and αIIbβ3 recognising the RGD tripeptide sequence, they are both fibronectin receptors. β5 is the beta component of only one integrin: αVβ5 which is known to bind osteopontin and vitronectin, its expression remained stable in chondrogenic condition but was initially downregulated followed by an upregulation in hMSCs cultured in growth medium. Once could think that this expression pattern would be similar for αV, however it is not the case as αV binds five different beta subunits.

The integrin subunit β1 expression was similar between hMSCs cultured in growth or in chondrogenic medium. Overall its expression remained stable, in pellet culture and collagen type II gels an initial downregulation followed by an upregulation was identified in both culture medium conditions. β1-integrins is a large family composed of 12 members: α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, α7β1, α8β1, α9β1, α10β1, α11β1, and αVβ1, they recognise numerous different ligands. It is thus not surprising to see little changes in its expression.

The only beta subunit were great expression changes were observed was β8, indeed its expression was importantly and continuously increased in hMSC cultured in growth medium. The β8 subunit associates with αV subunit to form αVβ8 integrins which is a receptor for vitronectin. We initially hypothesise that vitronectin adhesive properties are greatly increased via αVβ8 in hMSCs cultured in growth conditions however the expression of αV remained stable in growth conditions, further investigation with adhesion experiment would need to be performed in order to address this question.

5.4.5. Integrins Subunits Not Expressing During Chondrogenesis

As aforementioned we investigated expression of all the integrin subunits however several were not expressed during the chondrogenesis, those are α8, α9, αD, αE, αL, αM, αW and αX for the alpha subunits and β2, β4, β6 and β7 for the beta subunits.

β2-integrins are leukocytes-specific receptors, this subgroup includes αDβ2, αLβ2, αMβ2, and αXβ2, their expression is restricted to leukocytes hence it is normal that their expression was not detected in any of our culture system. The integrin αEβ7 is also a leukocyte-specific receptor.[2] Concerning the other integrin subunits not detected in our culture systems, including α8, α9, αW and β4, they were not expressed, to our knowledge, neither in other studies about hMSCs undergoing chondrogenesis. Indeed for instance, in Varas *et al.* study about integrin subunit during chondrogenic differentiation β2 and β6 were not detected neither.[63]

5.4.6. Future Work

Establishing integrins subunits transcript profile during chondrogenesis has provided a considerable knowledge about the adhesion requirements of the differentiating hMSCs but also about which integrins might play an important role to promote differentiation. Further investigations would need to done in order to assess the hypothesis about important integrins for chondrogenesis, disregulation of integrin signalling by using antibodies against specific subunit would provide useful information. Moreover, comparing the integrin subunits expression profile to expression in freshly isolated human chondrocytes might provide some interesting information. Additionally, the knowledge acquire about the adhesive properties of the differentiating hMSCs can be interpreted in order optimise biomaterials for cartilage tissue engineering from a chemical and biological perspective by presenting for instance a set of ligands according to the changing adhesion requirements of the hMSCs undergoing chondrogenesis.

.

	Expression changes in growth medium	Expression changes in chondrogenic medium	Integrin ligands affinity	Notes
ITGA1	Highly upregulated	Stable	**α1β1** ▪ primary chondrocyte receptor for collagen VI > collagen II ▪ affinity collagen IV > collagen I ▪ collagen IX	▪ Important variations in expression levels were observed. ▪ From literature: - Expression of ITGA1 decreased in aggregate culture.[63] - High expression on adult articular chondrocytes.[74] - Expression of ITGA1 Day 20/Day 0 was 0.63 in pellet culture.[75]
ITGA2	Stable	Highly downregulated	**α2β1** ▪ affinity collagen I > collagen IV ▪ collagen VI (weak) ▪ collagen IX	▪ Important variations in expression levels were observed. ▪ From literature: - Expression of ITGA2 unchanged in aggregate culture.[63] - ITGA2 is not significantly expressed in adult articular chondrocytes. Relative to adult chondrocytes α2β1 expression is higher in chondrosarcoma and foetal chondrocytes.[74] - Expression of ITGA2 Day 20/Day 0 was 1.3 in pellet culture.[75]
ITGA3	Depends on the method	Depends on the method	**α3β1** ▪ Laminin primarily ▪ Secondary receptor for collagen II and fibronectin ▪ Other ligands	▪ Time shift in micromass culture. ▪ From literature: - Expression of ITGA3 decreased in aggregate culture.[63] - High expression on adult articular chondrocytes.[74]
ITGA4	Depends on the method, relatively stable.	Depends on the method	**α4β1** ▪ Fibronectin predominantly ▪ Osteopontin ▪ Other ligands	▪ Time shift in micromass culture. ▪ From literature: - ITGA4 normally not expressed on adult articular chondrocytes.[74] - Expression of ITGA4 Day 20/Day 0 was 3.2 in pellet culture.[75]
ITGA5	Stable	Depends on the method	**α5β1** ▪ Primary chondrocyte receptor for fibronectin ▪ Osteopontin ▪ COMP ▪ Other irrelevant ligands	▪ Time shift in micromass culture. ▪ From literature: - Expression of ITGA5 unchanged in aggregate culture.[63] - High expression on adult articular chondrocytes.[74] - Expression of ITGA5 Day 20/Day 0 was 0.78 in pellet culture.[75]
ITGA6	Depends on the method	Depends on the method	**α6β1** ▪ Laminin	▪ Time shift in micromass culture. ▪ From literature: - α6β1 is not detected in all studies in adult articular chondrocytes. Relative to adult chondrocytes α6β1 expression is higher in chondrosarcoma and foetal chondrocytes.[74] - Expression of ITGA6 Day 20/Day 0 was 0.64 in pellet culture.[75]

Table 11 - Expression profile of integrin subunits and ligand-binding specificities of heterodimeric integrins

ITGA7	Unaffected by the culture medium. Exact same behaviour in control or in chondrogenic medium.		**α7β1** ▪ Laminin ▪ COMP	▪ From literature: - ITGA7 expression is mostly limited to white blood cells and satellite cells[2] - Expression of ITGA7 Day 20/Day 0 was 1.7 in pellet culture.[75]
ITGA10	Highly upregulated	Depends on the method	**α10β1** ▪ Collagen IV > Collagen VI > Collagen II ▪ Collagen IX ▪ Laminin	▪ Similar trend observed between growth and chondrogenic conditions however the upregulation was greater in growth conditions. ▪ From literature: - Expression of ITGA10 increased in aggregate culture.[63] - α10β1 is a primary collagen type II receptor in articular chondrocytes and is highly expressed in normal articular chondrocytes.[74]
ITGA11	Similar behaviour in control and chondrogenic conditions Moderately upregulated	Highly upregulated	**α11β1** ▪ Collagen I > Collagen IV ▪ Collagen IX	▪ ITGA11 was more expressed in chondrogenic conditions. ▪ From literature: - Expression of ITGA11 decreased in aggregate culture.[63]
ITGAV	Depends on the method	Depends on the method	**αVβ1, αVβ3, αVβ5, αVβ6, αVβ8** ▪ RGD receptors	▪ ITGAV was more expressed in chondrogenic conditions. ▪ From literature: - Expression of ITGAV remains unchanged in aggregate culture.[63] - αVβ5 is highly expressed in adult chondrocytes while αVβ3 is moderately expressed. - Expression of ITGAV Day 20/Day 0 was 0.81 in pellet culture.[75]
ITGB1	Relatively stable. Similar behaviour in control and in chondrogenic medium.		**α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, α7β1, α8β1, α9β1, α10β1, α11β1, and αVβ1.** ▪ See the alpha subunits for ligand affinity of the abovementioned integrins	▪ Time shift in micromass culture. ▪ From literature: - Expression of ITGB1 remains unchanged in aggregate culture.[63] - Expression of ITGAV Day 20/Day 0 was 0.5 in pellet culture.[75]
ITGB3	Stable	Initial plateau, followed by a downregulation from Day 14 onwards	**αVβ1 and αIIbβ1**	▪ From literature: - Expression of ITGB3 remains unchanged in aggregate culture.[63]
ITGB5	Downregulation followed by upregulation	Relatively stable	**αVβ5**	▪ From literature: - Expression of ITGB5 remains unchanged in aggregate culture.[63] - Expression of ITGAV Day 20/Day 0 was 5.2 in pellet culture.[75]
ITGB8	Very important upregulation	Relatively stable	**αVβ8**	▪ Very important variations in expression levels were observed

6. Conclusion and Prospect

In the present work, we investigated integrin subunit transcript expression during hMSC chondrogenesis in pellet culture, micromass culture and collagen type II hydrogels. We were able to establish an expression profile over time for each integrin subunit according to the culture system.

The chondrocyte phenotype markers expression analysis along with the histology staining revealed that the system where the most ECM was produced was pellet culture. However the data suggests that fibrocartilage is produced and that chondrocytes in pellet culture might eventually undergo hypertrophy as a final stage of differentiation. On the other hand, hMSCs undergoing chondrogenesis in collagen type II gels revealed the best phenotype, yet one should stimulate the production of ECM in order to make this culture system superior to the two other systems investigated.

We demonstrated that integrins are overall more expressed in hMSCs cultured in growth conditions than in hMSCs undergoing chondrogenic differentiation. Nonetheless integrin subunits α11 and αV were more expressed in chondrogenic conditions and might thus play an important role to direct stem cell differentiation toward chondrocytes. Furthermore, we identified identical expression profiles over time for the alpha subunits related to collagen-binding integrins irrespective to the culture systems, while the profile of fibronectin- and laminin-binding receptors was variable and seemed dependent on the culture method.

This project was the first step of a larger study which seeks to elucidate the role of integrins during chondrogenic differentiation. Thus the initial step was to establish integrin subunits transcript expression in order to carry out further investigations. The next step is to link the observed integrin expression in this study to the role of integrins in driving chondrogenesis. For instance, disregulation of integrin signalling with antibodies could prove that the integrin subunits we quantified are important regulators of chondrogenesis. More interestingly, the huge data collected during this project can be interpreted in order to present the right ligand over time to promote differentiation and stabilise chondrocyte phenotype. One could imagine to seed hMSCs in 3D hydrogel where an integrin receptor ligand for hMSCs would be presented and over time, the desired ligands for chondrocytes would become available.

In conclusion, this work has led to an improved understanding of the expression of integrins in hMSCs and during chondrogenesis and this knowledge can be used to design biomaterial that mimics endogenous integrin binding pattern as reproducing integrin temporal expression could significantly improve chondrogenic differentiation in tissue engineered constructs.

7. Appendix

7.1. qPCR for Primer Validation

7.1.1. ITGA1 v2

Efficiency	R^2	Threshold
0.98	0.94021	0.1496

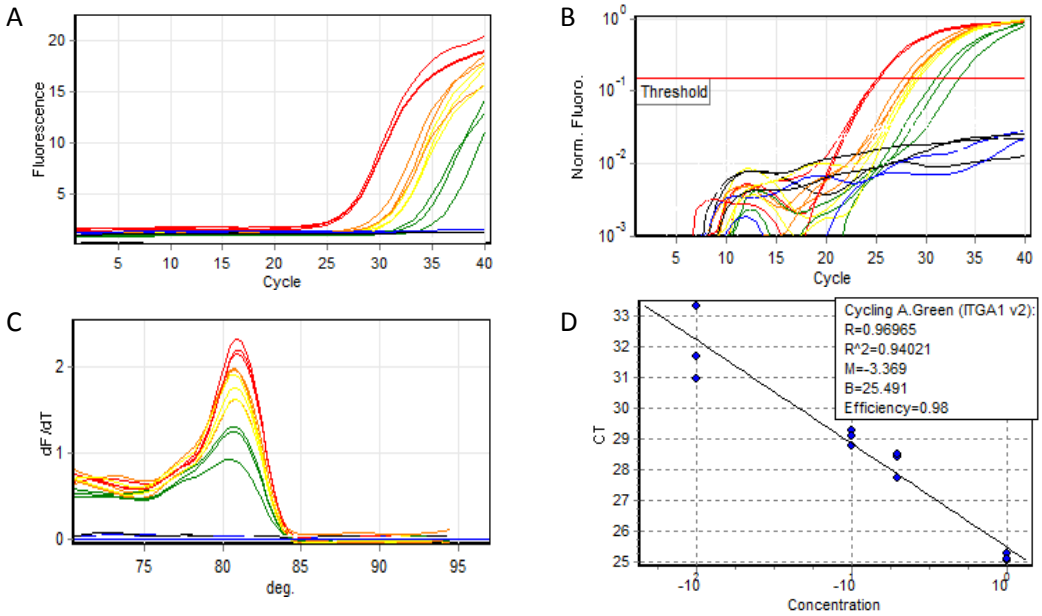


Figure 29 - Primer validation for ITGA1. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.2. ITGA2 v1

Efficiency	R^2	Threshold
0.93	0.99659	0.1958

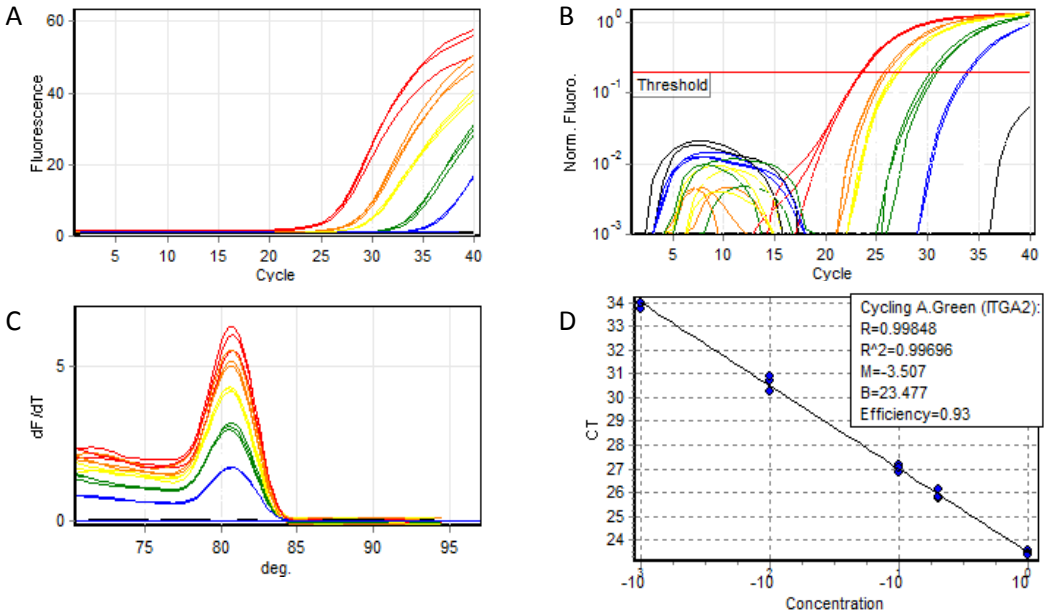


Figure 30 - Primer validation for ITGA2. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.3. ITGA3 v3

Efficiency	R^2	Threshold
1.00	0.98731	0.1498

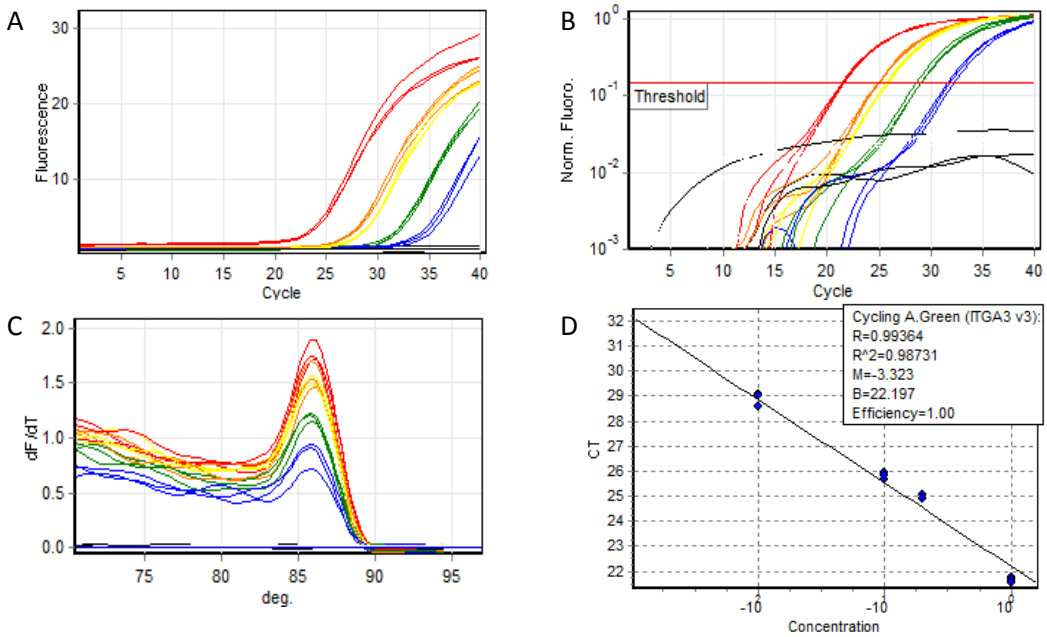


Figure 31 **-** Primer validation for ITGA3. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.4. ITGA4 QIAGEN

Efficiency	R^2	Threshold
1.01	0.9896	0.1321

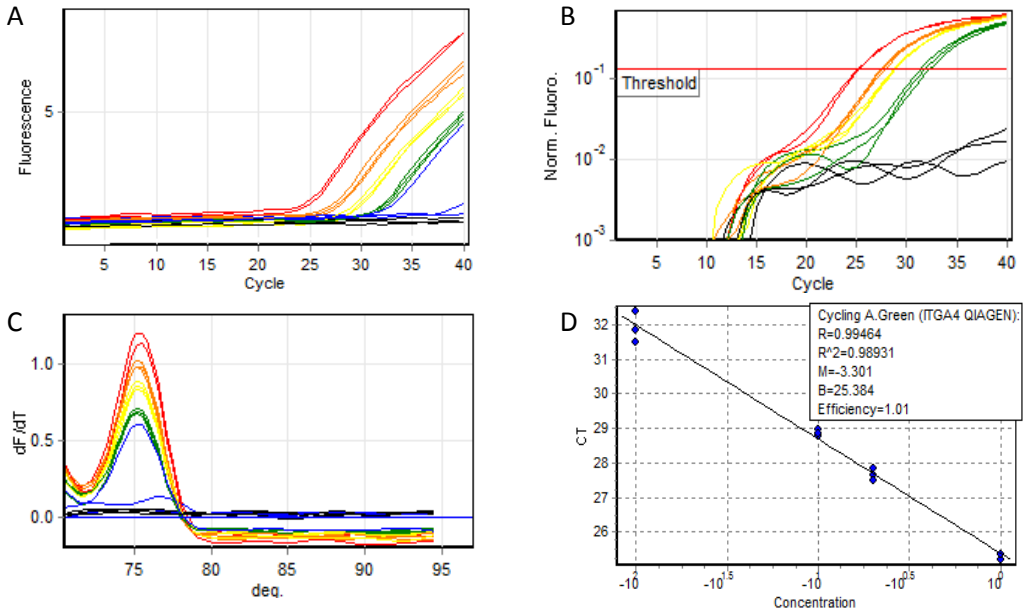


Figure 32 - Primer validation for ITGA4. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.5. ITGA5 v1

Efficiency	R^2	Threshold
1.00	0.99695	0.0838

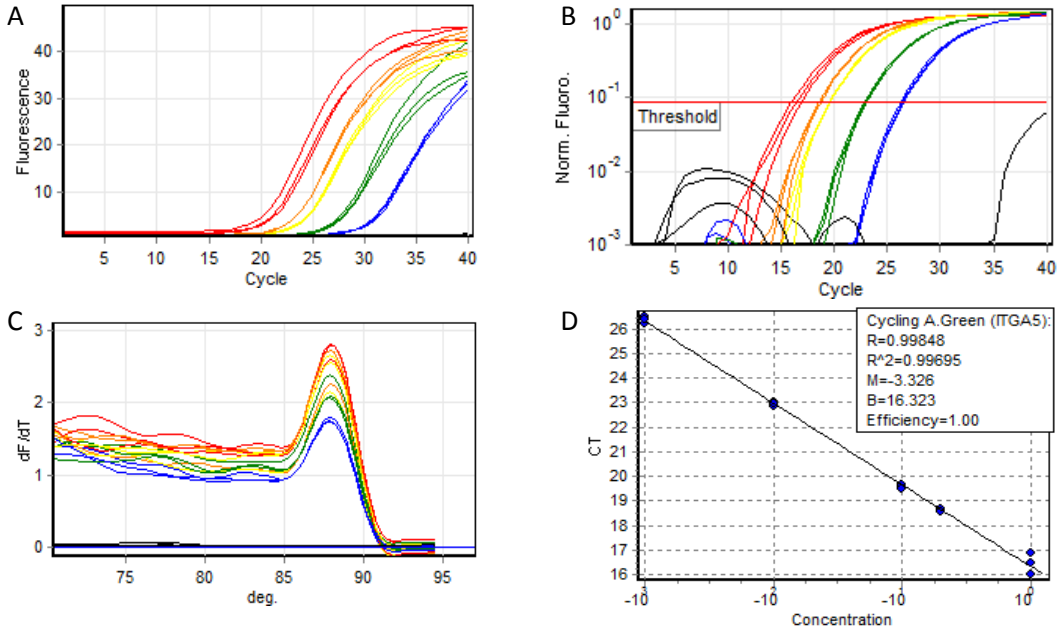


Figure 33 - Primer validation for ITGA5. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.6. ITGA6 QIAGEN

Efficiency	R^2	Threshold
0.9	0.9694	0.145

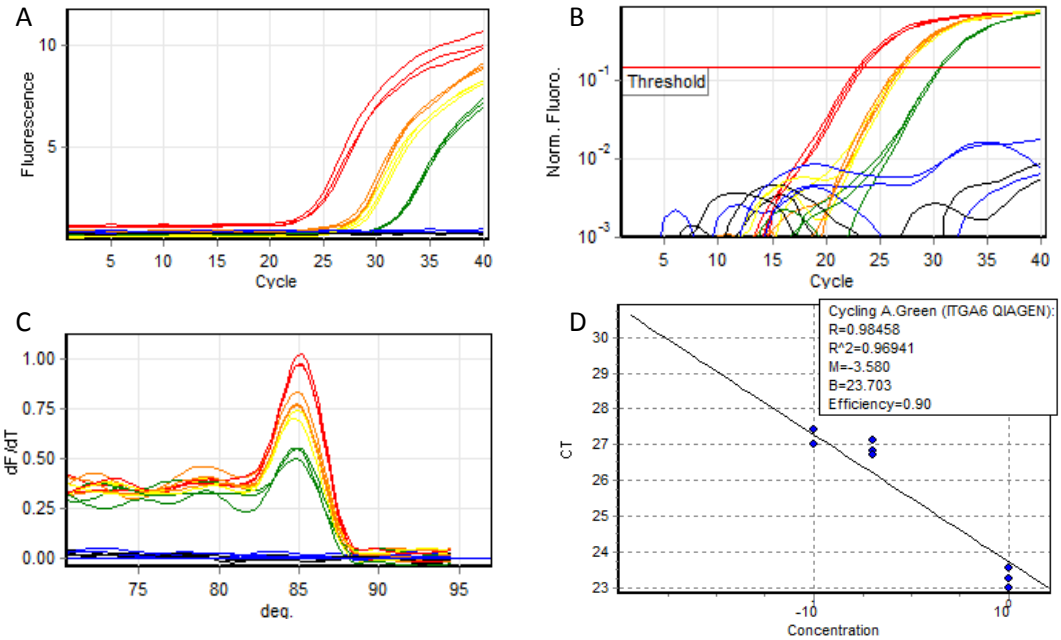


Figure 34 - Primer validation for ITGA6. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.7. ITGA7 v2

Efficiency	R^2	Threshold
0.98	0.97420	0.03

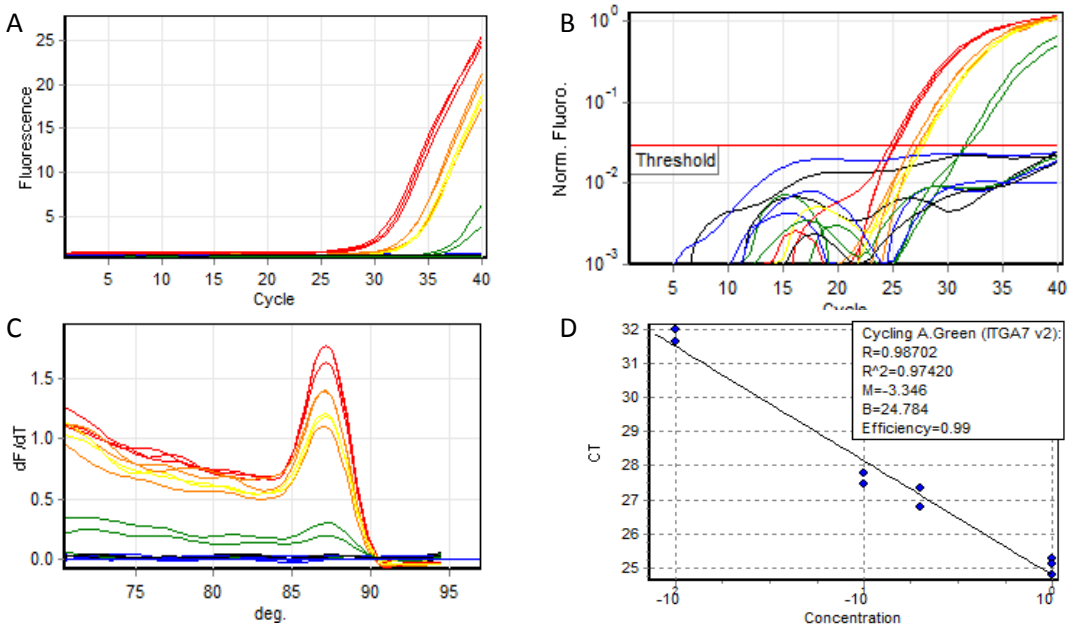


Figure 35 - Primer validation for ITGA7. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.8. ITGA10 v1

Efficiency	R^2	Threshold
1.00	0.9968	0.1340

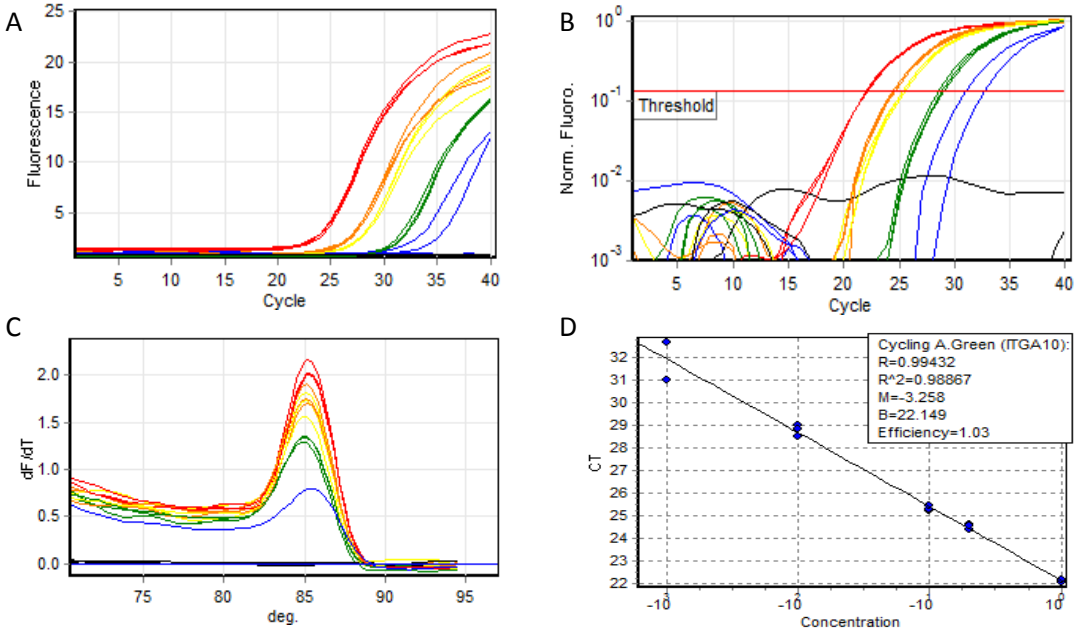


Figure 36 - Primer validation for ITGA10. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.9. ITGA11 v1

Efficiency	R^2	Threshold
1.09	0.97012	0.03

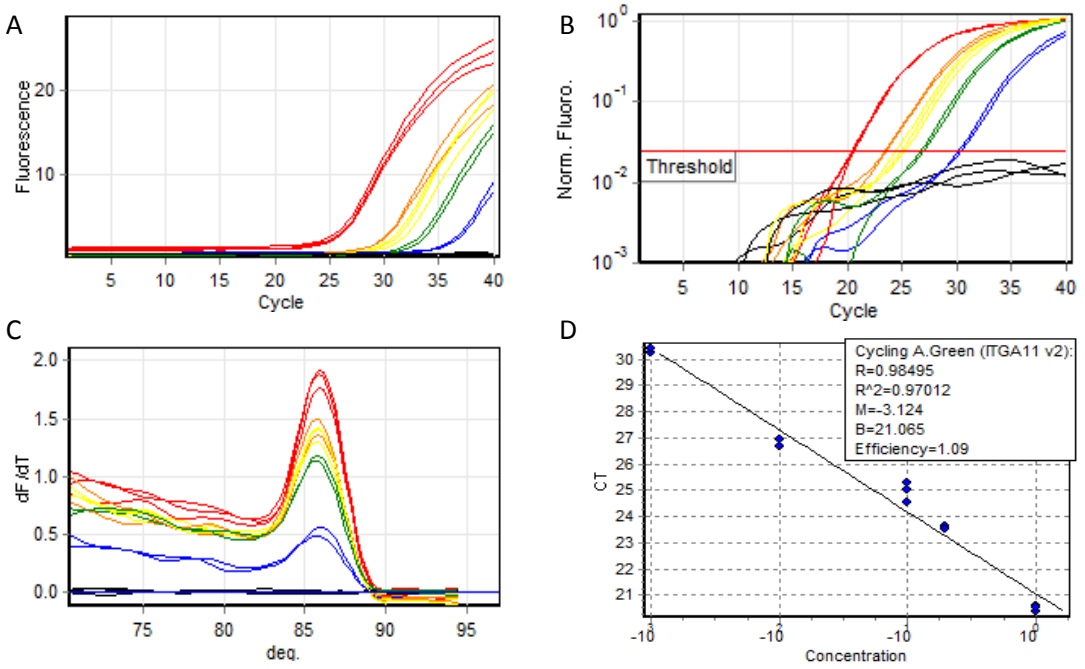


Figure 37 - Primer validation for ITGA11. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.10. ITGAV v1

Efficiency	R^2	Threshold
0.96	0.9921	0.0712

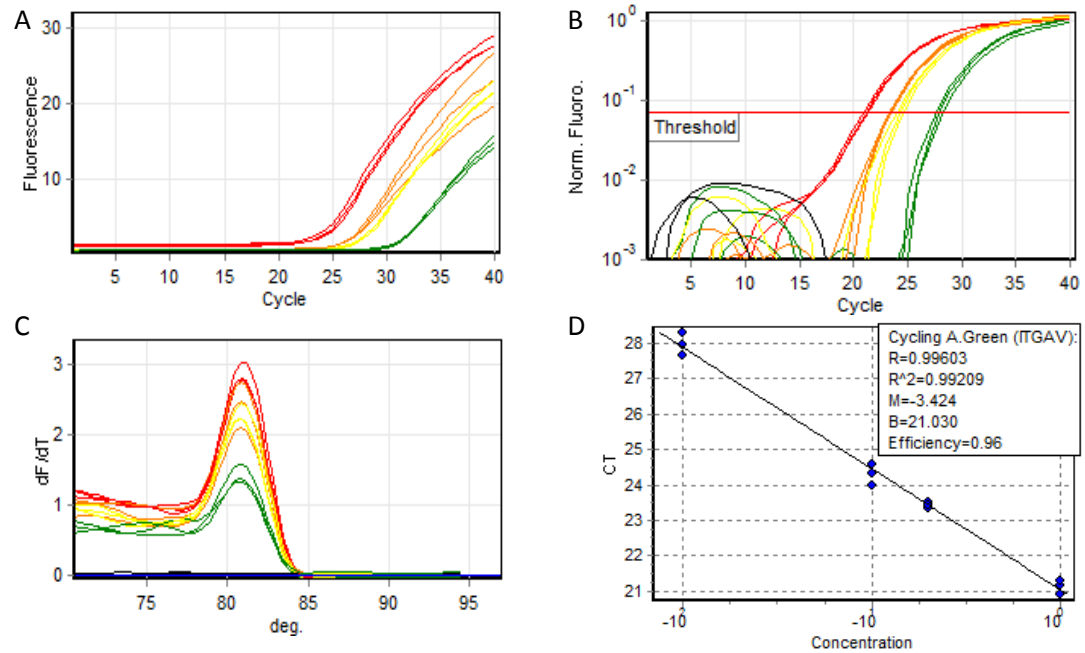


Figure 38 - Primer validation for ITGAV. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.11. ITGB1 v2

Efficiency	R^2	Threshold
0.96	0.9991	0.1229

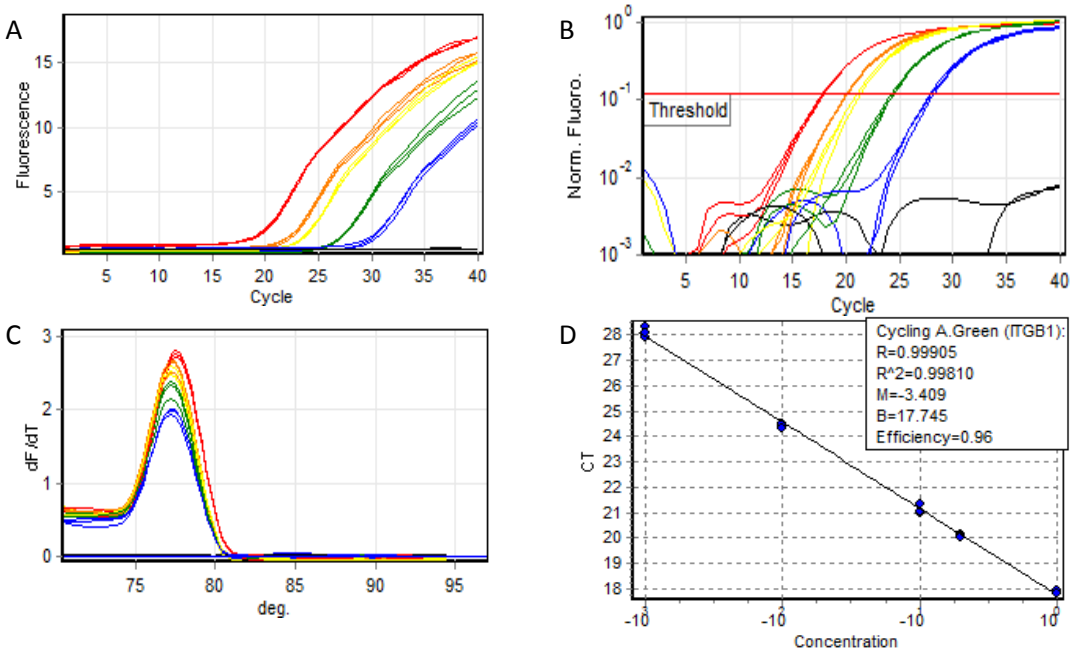


Figure 39 - Primer validation for ITGB1. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.12. ITGB3 v1

Efficiency	R^2	Threshold
0.99	0.9963	0.02

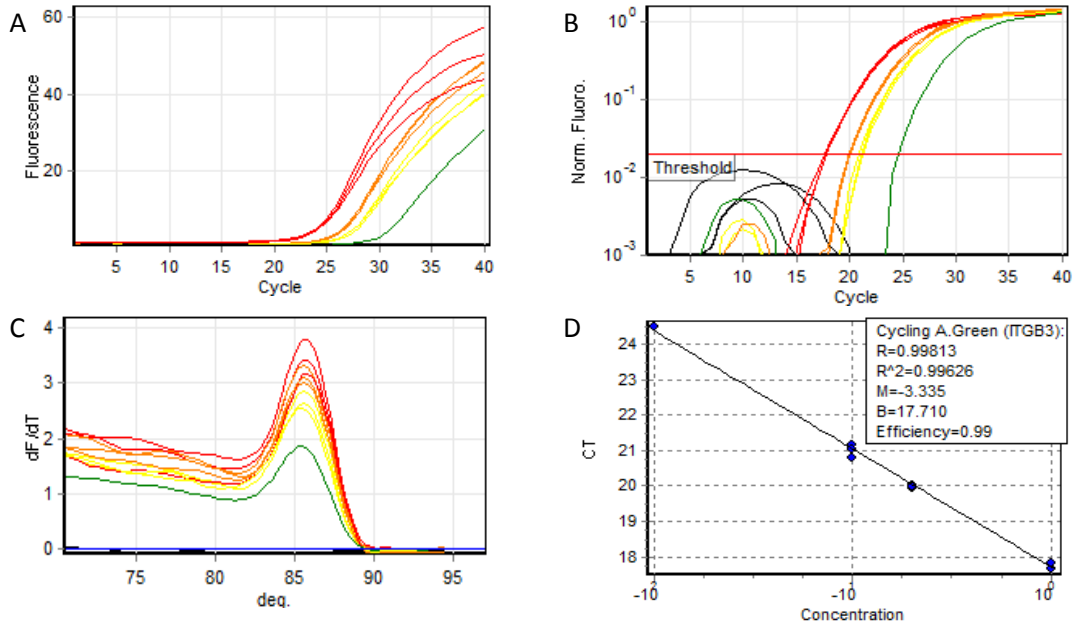


Figure 40 - Primer validation for ITGB3. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.13. ITGB5 v2

Efficiency	R^2	Threshold
0.98	0.9676	0.1498

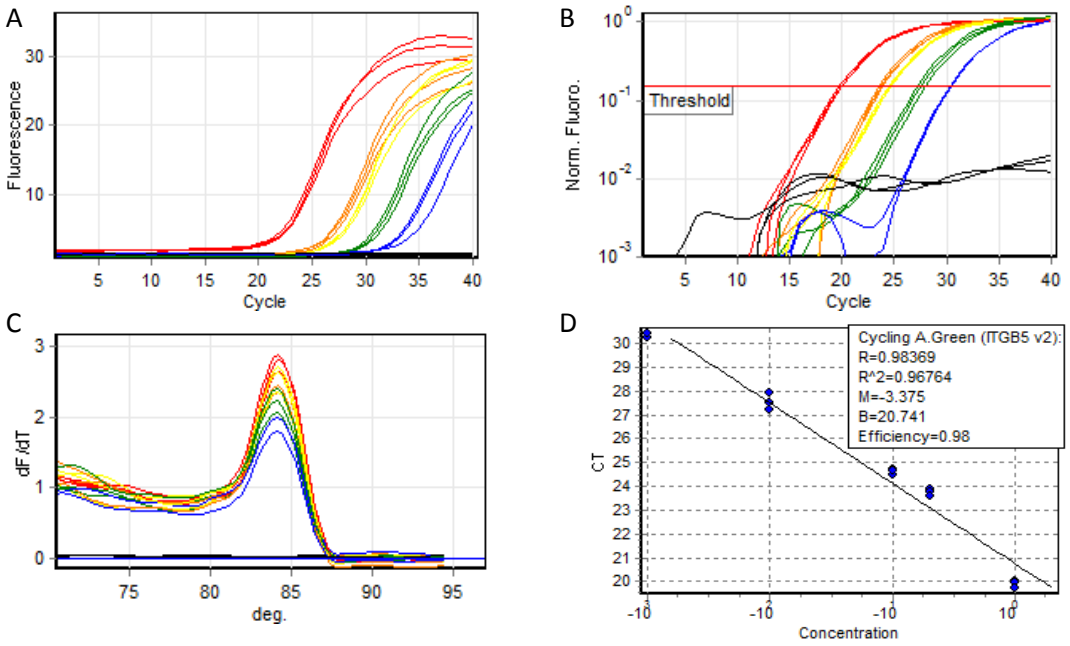


Figure 41 - Primer validation for ITGB5. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.14. ITGB8 v2

Efficiency	R^2	Threshold
0.99	0.9709	0.1268

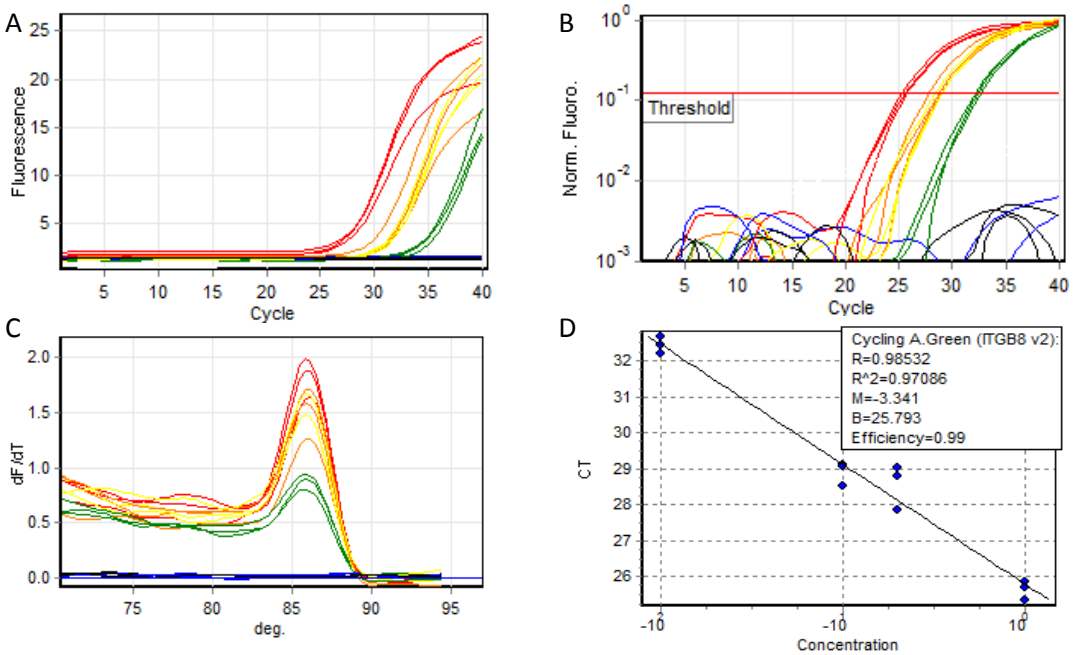


Figure 42 **-** Primer validation for ITGB8. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.15. ACAN

Efficiency	R^2	Threshold
0.91	0.9895	0.04

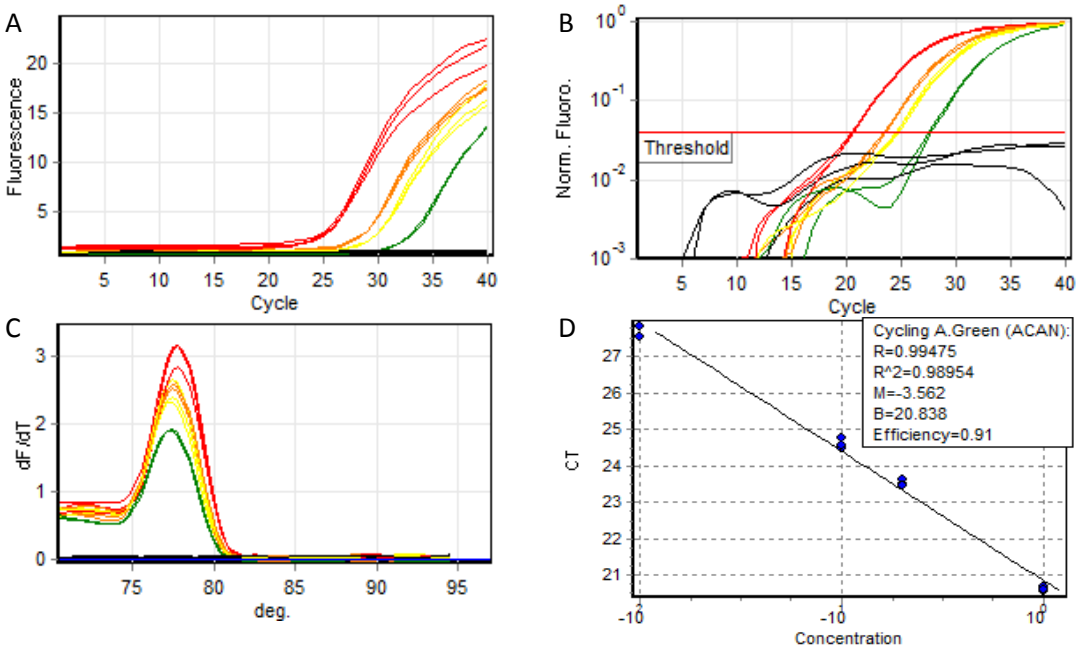


Figure 43 - Primer validation for ACAN. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.16. COL1A1

Efficiency	R^2	Threshold
0.93	0.9987	0.1472

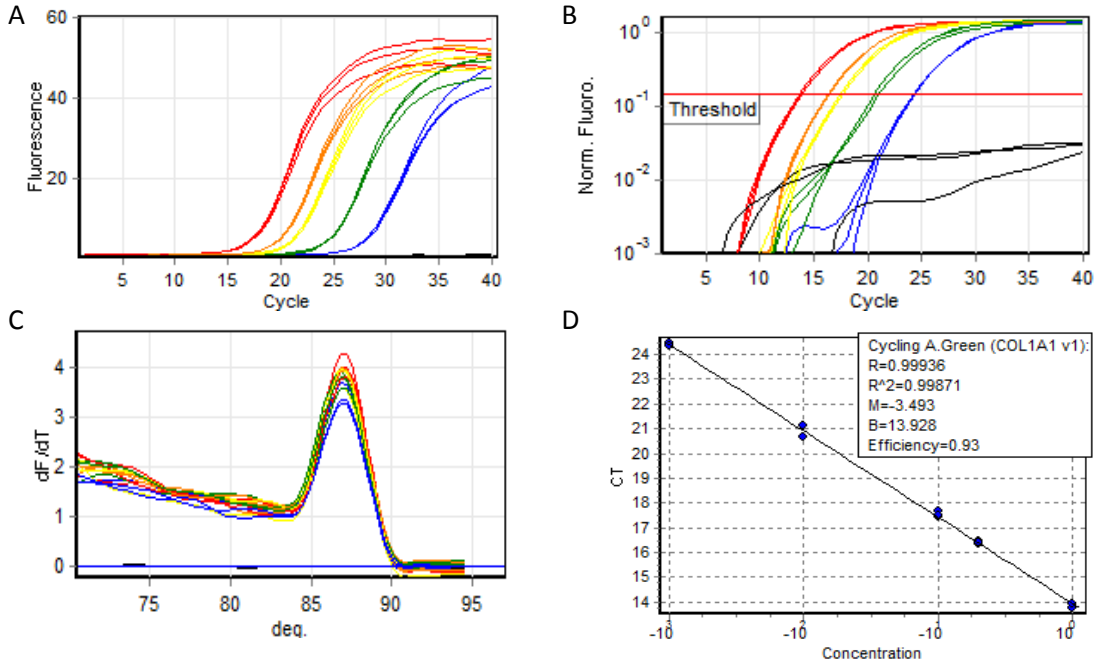


Figure 44 - Primer validation for COL1A1. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.17. COL2A1

Efficiency	R^2	Threshold
1.00	0.9973	0.137

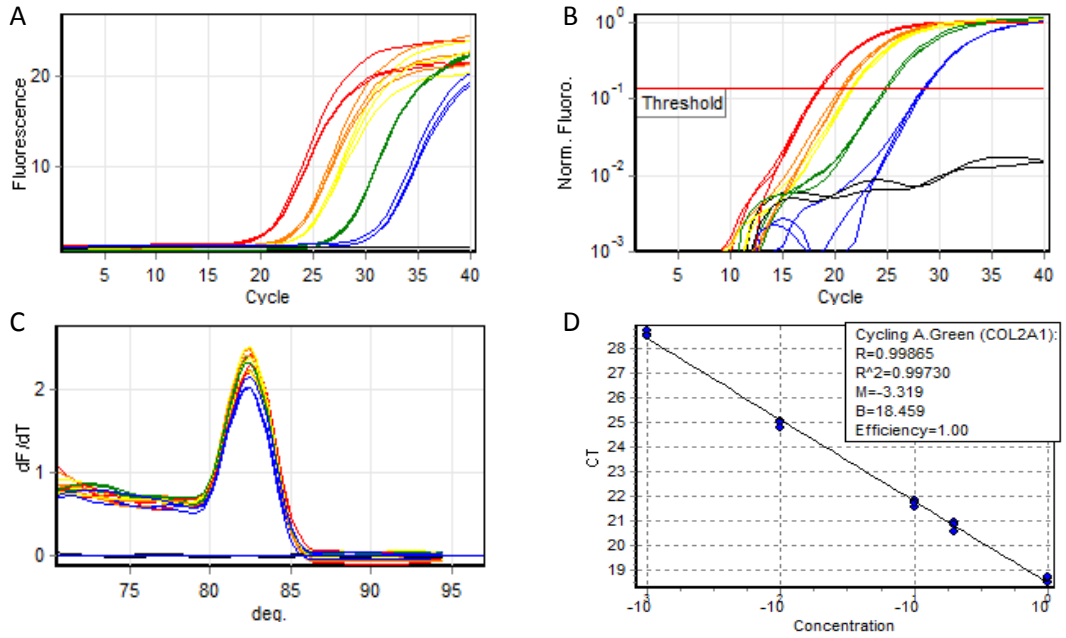


Figure 45 - Primer validation for COL2A1. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.18. COL10A1

Efficiency	R^2	Threshold
1.01	0.9661	0.0344

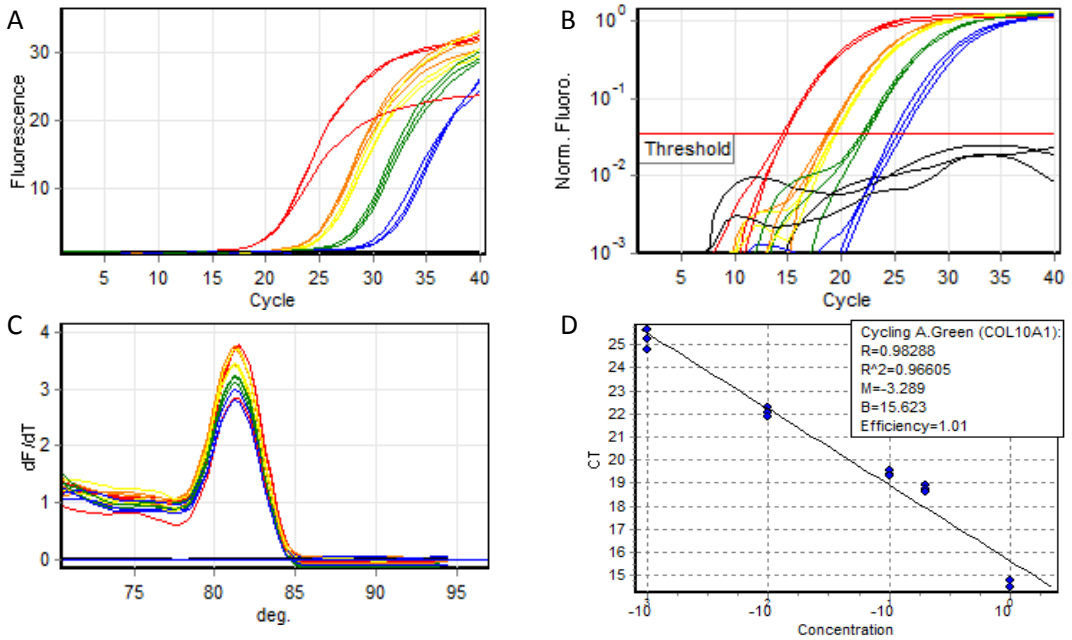


Figure 46 **-** Primer validation for COL10A1. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.19. GAPDH

Efficiency	R^2	Threshold
0.97	0.9926	0.1498

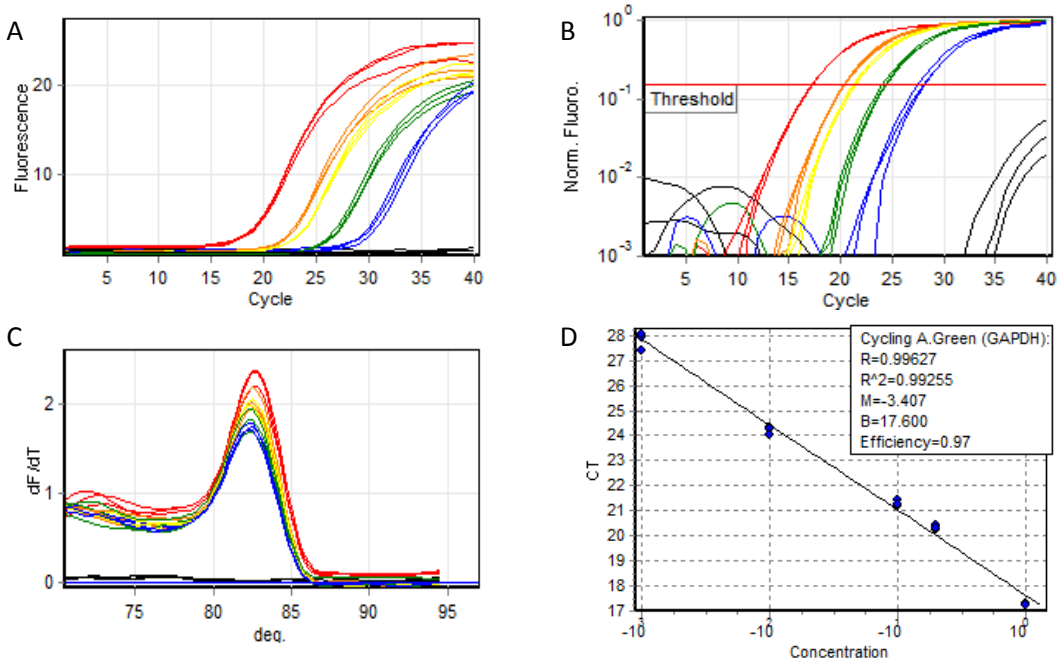


Figure 47 - Primer validation for GAPDH. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.20. RUNX2

Efficiency	R^2	Threshold
1.05	0.99293	0.1498

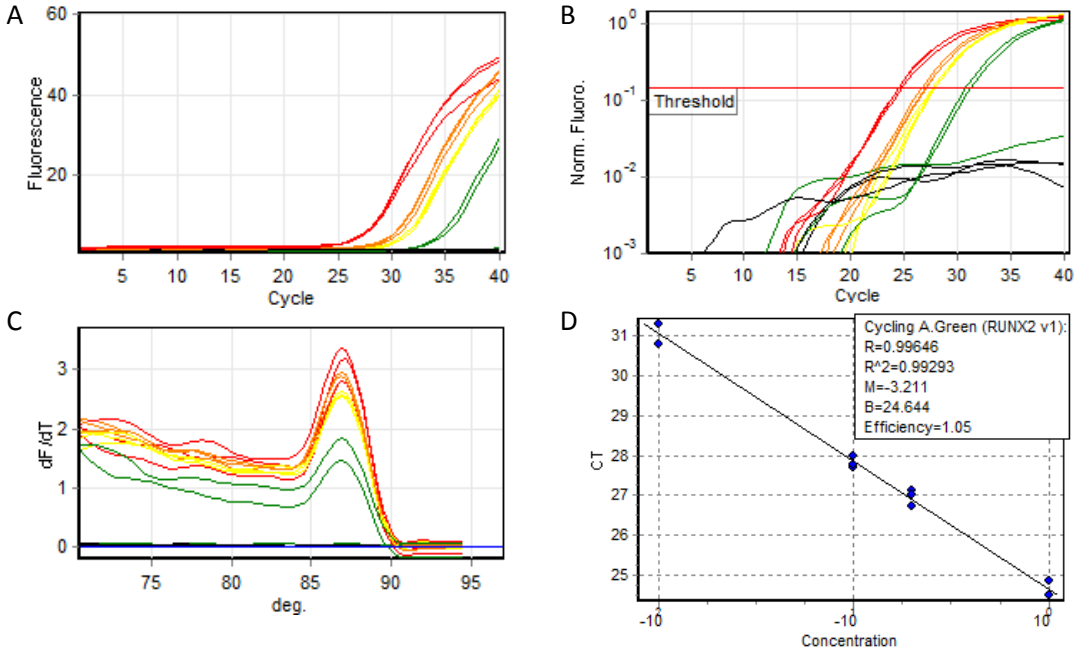


Figure 48 - Primer validation for RUNX2. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.1.21. SOX9

Efficiency	R^2	Threshold
0.9	0.9936	0.059

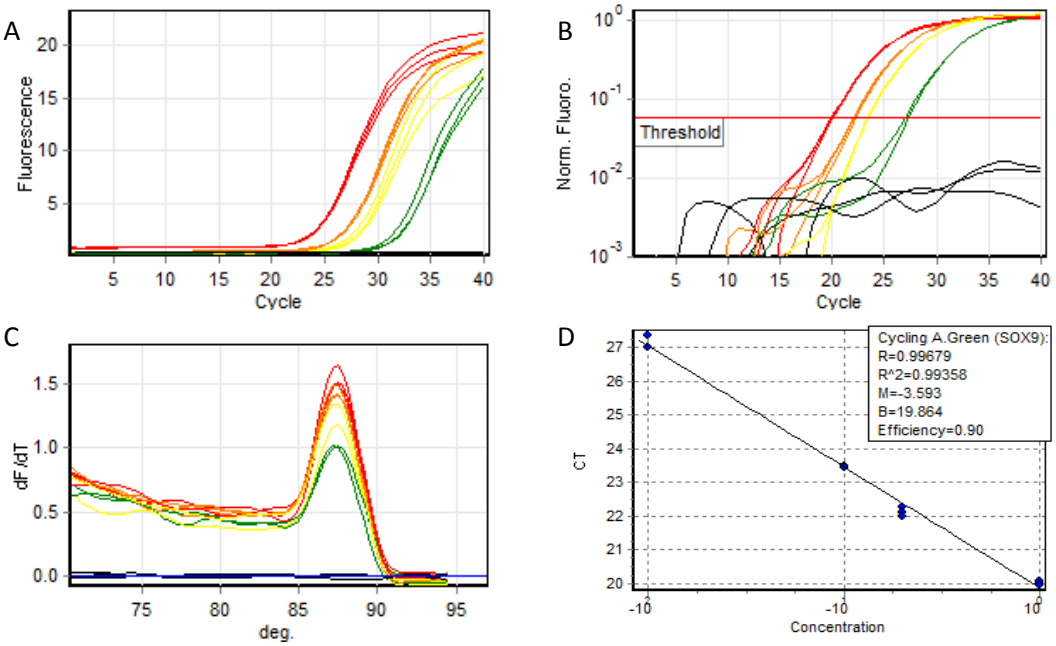
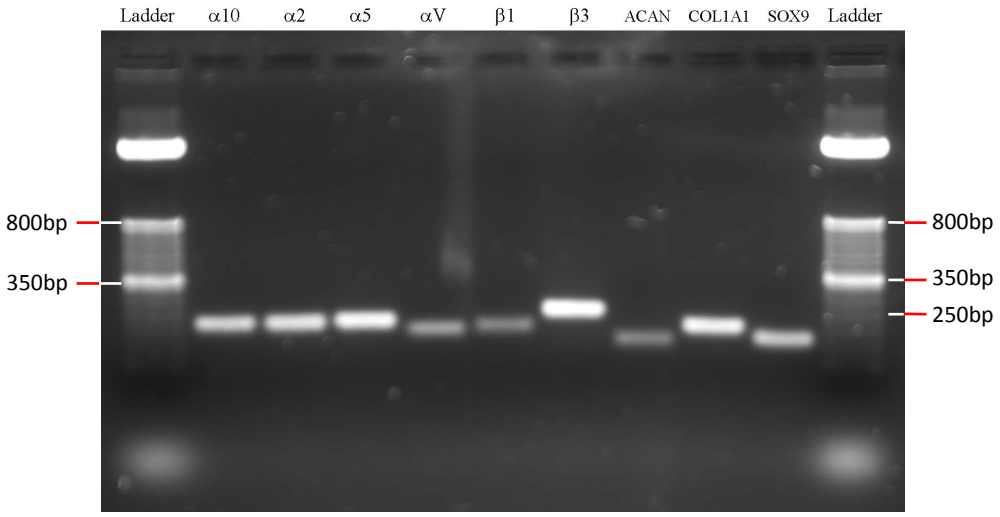
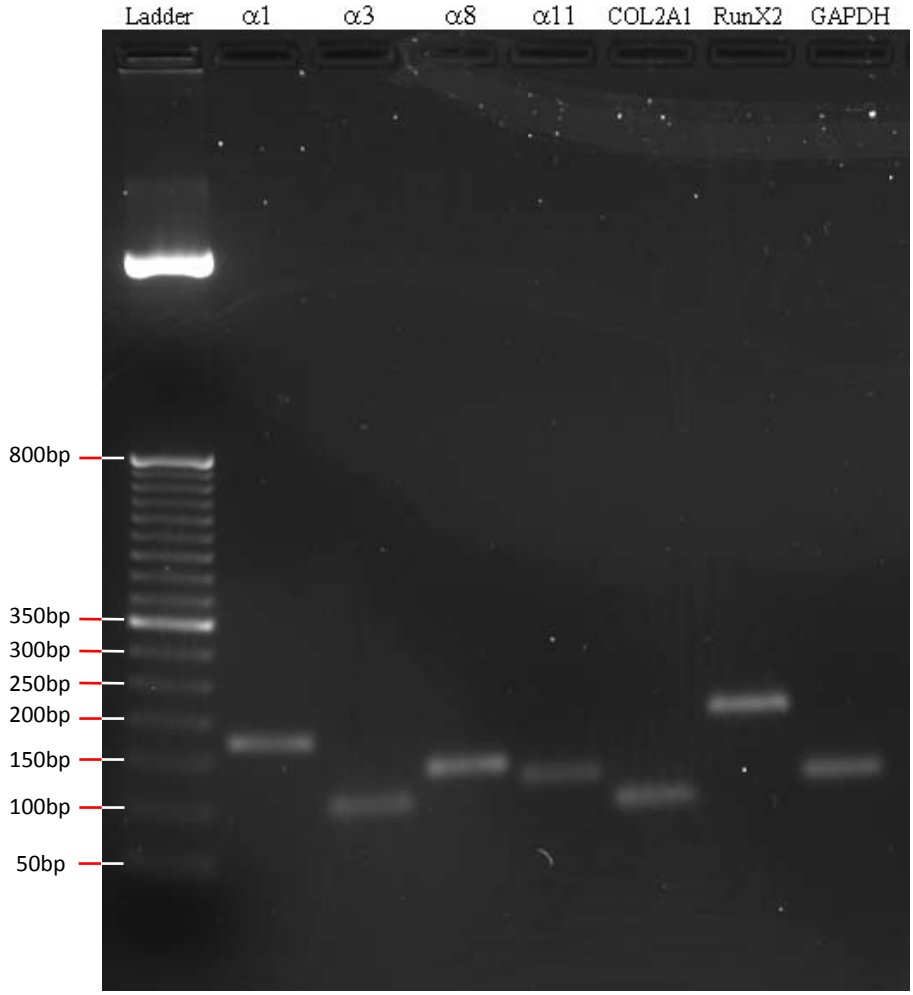
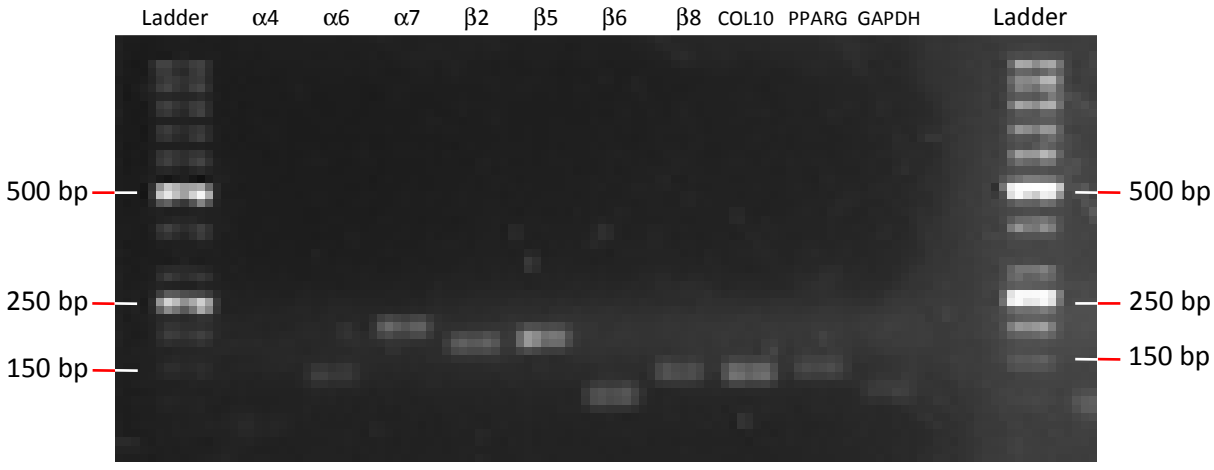


Figure 49 **-** Primer validation for SOX9. In order to get a standard curve, the qPCR was performed in technical triplicates with a serial dilution ofcDNA from hMSCs cultured on tissue culture plastic, dilutions were 1:10, 1:50, 1:100, 1:1000 and 1:10000. Amplification curves (A), quantification curves and threshold (B), melt curve (C), and standard curve (D).

7.2. Gel Electrophoresis UV Pictures




Ladder
α1
α3
α8
α11
COL2A1
RunX2
GAPDH
800bp
350bp
300bp
250bp
200bp
150bp
100bp
50bp


Ladder
α4
α6
α7
β2
β5
β6
β8
COL10
PPARG
GAPDH
Ladder
500 bp
250 bp
150 bp
500 bp
250 bp
150 bp

8. References

1 Abram, C. L. & Lowell, C. A. The ins and outs of leukocyte integrin signaling. *Annu Rev Immunol* **27**, 339-362 (2009).

2 Hynes, R. O. Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-687 (2002).

3 Guilak, F. *et al.* The pericellular matrix as a transducer of biomechanical and biochemical signals in articular cartilage. *Ann N Y Acad Sci* **1068**, 498-512 (2006).

4 James, C. B. & Uhl, T. L. A review of articular cartilage pathology and the use of glucosamine sulfate. *J Athl Train* **36**, 413-419 (2001).

5 Mow, V. C. & Huiskes, R. *Book basic orthopaedic biomechanics & mechano-biology*. 3rd edition edn, 736 (Lippincott Williams & Wilkins, 2005).

6 Alexopoulos, L., Setton, L. & F., G. The biomechanical role of the chondrocyte pericellular matrix in articular cartilage. *Acta Biomaterialia* **1**, 317-325 (2005).

7 Gemmiti, C. V. & Guldberg, R. E. Fluid flow increases type II collagen deposition and tensile mechanical properties in bioreactor-grown tissue-engineered cartilage. *Tissue Eng.* **12**, 469-479 (2006).

8 Miyanishi, K. *et al.* Effects of hydrostatic pressure and transforming growth factor-beta 3 on adult human mesenchymal stem cell chondrogenesis in vitro. *Tissue Eng.* **12**, 1419-1428 (2006).

9 Scherer, K., Schünke, M., Sellckau, R., Hassenpflug, J. & Kurz, B. The influence of oxygen and hydrostatic pressure on articular chondrocytes and adherent bone marrow cells in vitro. *Biorheology* **41**, 323-333 (2004).

10 De Croos, J. N., Dhaliwal, S. S., Grynpas, M. D., Pilliar, R. M. & Kandel, R. A. Cyclic compressive mechanical stimulation induces sequential catabolic and anabolic gene changes in chondrocytes resulting in increased extracellular matrix accumulation.

11 Waldman, S. D., Couto, D. C., Grynpas, M. D., Pilliar, R. M. & Kandel, R. A. A single application of cyclic loading can accelerate matrix deposition and enhance the properties of tissue-engineered cartilage. *Osteoarthritis Cartilage* **14**, 323-330 (2006).

12 Meyer, E., Buckley, C. T., Thorpe, S. D. & Kelly, D. J. Low oxygen tension is a more potent promoter of chondrogenic differentiation than dynamic compression. *J Biomech* **43**, 2516-2523 (2010).

13 Park, Y., Sugimoto, M., Watrin, A., Chiquet, M. & Hunziker, E. B. BMP-2 induces the expression of chondrocyte-specific genes in bovine synovium-derived progenitor cells cultured in three-dimensional alginate hydrogel. *Osteoarthr Cartil* **13**, 527-536, doi:10.1016/j.joca.2005.02.006 (2005).

14 Barry, F., Boynton, R. E., Liu, B. & Murphy, J. M. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. *Exp Cell Res* **268**, 189-200, doi:10.1006/excr.2001.5278 (2001).

15 Li, H., Feng, F., Bingham III, C. O. & Elisseeff, J. H. Matrix metalloproteinases and inhibitors in cartilage tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine* (2011).

16 Cawston, T. *et al.* The regulation of MMPs and TIMPs in cartilage turnover. *Annals of the New York Academy of Sciences* **878**, 120-129 (1999).

17 Chung, C. & Burdick, J. A. Engineering Cartilage Tissue. *Advanced Drug Delivery Reviews* **60**, 243-262 (2008).

18 Watanabe, H., Yamada, Y. & Kimata, K. Roles of aggrecan, a large chondroitin sulfate proteoglycan, in cartilage structure and function. *J Biochem* **124**, 687-693 (1998).

19 Roughley, P. & Lee, E. Cartilage proteoglycans: structure and potential functions. *Microscopy Research and Technique* **28**, 385-397 (1994).

20 Knudson, C. B. & Knudson, W. Cartilage proteoglycans. *Semin Cell Dev Biol* **12**, 69-78 (2001).

21 Darling, E. M. & Athanasiou, K. A. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *Journal of Orthopaedic Research* **23**, 425-432 (2005).
22 Keeney, M. & Lai, J. H. Y., F. Recent progress in cartilage tissue engineering. *Curr Opin Biotechnol* (2011).
23 Koga, H., Engebretsen, L., Brinchmann, J. E., Muneta, T. & Sekiya, I. Mesenchymal stem cell-based therapy for cartilage repair: a review. *Knee Surg Sports Traumatol Arthrosc* **17**, 1289-1297, doi:10.1007/s00167-009-0782-4 (2009).
24 Fernandes, J. C., Martel-Pelletier, J. & Pelletier, J. P. The role of cytokines in osteoarthritis pathophysiology. *Biorheology* **39**, 237-246 (2002).
25 Seed, S. M., Dunican, K. C. & Lynch, A. M. Osteoarthritis: a review of treatment options. *Geriatrics* **64**, 20-29 (2009).
26 Cheng, Y. J., Hootman, J. M., Murphy, L. B., Langmaid, G. A. & Helmick, C. G. Prevalence of Doctor-Diagnosed Arthritis and Arthritis-Attributable Activity Limitation - United States, 2007-2009. *MMWR* **59**, 1261-1265 (2010).
27 Pelttari, K., Steck, E. & Richter, W. The use of mesenchymal stem cells for chondrogenesis. *Injury* **39**, S58-65 (2008).
28 Steinwachs, M., Guggi, T. & Kreuz, P. Marrow stimulation techniques. *Injury* **39**, S26-31 (2008).
29 Brittberg, M. *et al.* Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *The New England Journal of Medicine* **331**, 889-895 (1994).
30 Harris, J. D., Siston, R. A., Pan, X. & Flanigan, D. C. Autologous chondrocyte implantation: a systematic review. *The Journal of Bone and Joint Surgery* **92**, 2220-2233, doi:10.2106/JBJS.J.00049 (2010).
31 Johnstone, B., Hering, T. M., Caplan, A. I., Goldberg, V. M. & Yoo, J. U. In Vitro Chondrogenesis of Bone Marrow-Derived Mesenchymal Progenitor Cells. *Experimental Cell Research* **39**, 265-272 (1998).
32 Ashton, B. A. *et al.* Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo. *Clin Orthop Relat Res.*, 294-307 (1980).
33 Sekiya, I., Larson, B. L., Vuoristo, J. T., Reger, R. L. & Prockop, D. J. Comparison of effect of BMP-2, -4, and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma. *Cell Tissue Res.* **320**, 269-276 (2005).
34 Haynesworth, S. E., Goshima, J., Goldberg, V. M. & Caplan, A. I. Characterization of cells with osteogenic potential from human marrow. *Bone* **13**, 81-88 (1992).
35 Pittenger, M. F. *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143-147 (1999).
36 Awad, H. A. *et al.* Autologous mesenchymal stem cell-mediated repair of tendon. *Tissue Eng* **5**, 267-277 (1999).
37 Mezey, E., Chandross, K. J., Harta, G., Maki, R. A. & McKercher, S. R. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science* **290**, 1779-1982 (2000).
38 Petersen, B. E. *et al.* Bone Marrow as a Potential Source of Hepatic Oval Cells. *Science* **284**, 1168-1170 (1999).
39 Orlic, D. *et al.* Bone marrow cells regenerate infarcted myocardium. *Nature* **410**, 701-705 (2001).
40 De Bari, C., Dell'Accio, F., Tylzanowski, P. & Luyten, F. P. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum* **44**, 1928-1942 (2001).
41 Cao, B. *et al.* Muscle stem cells differentiate into haematopoietic lineages but retain myogenic potential. *Nat Cell Biol* **5**, 640-646 (2003).
42 Zuk, P. A. *et al.* Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279-4295 (2002).

43 Fukumoto, T. *et al.* Combined effects of insulin-like growth factor-1 and transforming growth factor-beta1 on periosteal mesenchymal cells during chondrogenesis in vitro. *Osteoarthr Cartil* **11**, 55-64 (2003).
44 Sakaguchi, Y., Sekiya, I., Yagishita, K. & Muneta, T. Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis Rheum* **52**, 2521-2529, doi:10.1002/art.21212 (2005).
45 Huang, J. I. *et al.* Chondrogenic potential of progenitor cells derived from human bone marrow and adipose tissue: a patientmatched comparison. *J Orthop Res* **23**, 1383-1389 (2005).
46 Alhadlaq, A. & Mao, J. J. Mesenchymal stem cells: isolation and therapeutics. *Stem Cells Dev* **13**, 436-448 (2004).
47 Gronthos, S. *et al.* Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells. *J Bone Miner Res.* **14**, 47-56 (1999).
48 Hung, S. C. *et al.* Isolation and characterization of size-sieved stem cells from human bone marrow. *Stem Cells* **20**, 249-258 (2002).
49 Mauck, R. L., Yuan, X. & Tuan, R. S. Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture. *Osteoarthr Cartil* **14**, 179-189, doi:10.1016/j.joca.2005.09.002 (2006).
50 Steck, E. *et al.* Induction of intervertebral disc-like cells from adult mesenchymal stem cells. *Stem Cells* **23**, 403-411, doi:10.1634/stemcells.2004-0107 (2005).
51 Pelttari, K. *et al.* Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. *Arthritis Rheum* **54**, 3254-3266, doi:10.1002/art.22136 (2006).
52 Ichinose, S., Yamagata, K., Sekiya, I., Muneta, T. & Tagami, M. Detailed examination of cartilage formation and endochondral ossification using human mesenchymal stem cells. *Clinical and Experimental Pharmacology and Physiology* **32**, 561-570 (2005).
53 Zhang, X. *et al.* Primary murine limb bud mesenchymal cells in long-term culture complete chondrocyte differentiation: TGF-beta delays hypertrophy and PGE2 inhibits terminal differentiation. *Bone* **34**, 809-817 (2004).
54 Meredith, J. E., Fazeli, B. & Schwartz, M. A. The extracellular matrix as a cell survival factor. *Mol Biol Cell* **4**, 953-961 (1993).
55 Daniels, K. S., M. Modulation of chondrogenesis by the cytoskeleton and extracellular matrix. *J Cell Sci* **100**, 249-254 (1991).
56 Barczyk, M., Carracedo, S. & Gullberg, D. Integrins. *Cell Tissue Res* **339**, 269-280, doi:10.1007/s00441-009-0834-6 (2010).
57 Humphries, M. J. & Symonds, E. J. M., A. P. Mapping functional residues onto integrin crystal structures. *Curr Opin Struct Biol* **13**, 236-243 (2003).
58 Nievers, M. G., Schaapveld, R. Q. & Sonnenberg, A. Biology and function of hemidesmosomes. *Matrix Biology* **18**, 5-17 (1999).
59 Geiger, B., Spatz, J. P. & Bershadsky, A. D. Environmental sensing through focal adhesions. *Nat Rev Mol Cell Biol* **10**, 21-33 (2009).
60 Loeser, F. R. Integrins and cell signaling in chondrocytes. *Biorheology* **39**, 119-124 (2002).
61 Solursh, M. L., T. F. Jensen, K. L. Chondrogenesis from single limb mesenchyme cells. *Developmental Biology* **94**, 259-264 (1982).
62 Zaucke, F., Dinser, R., Maurer, P. & Paulsson, M. Cartilage oligomeric matrix protein (COMP) and collagen IX are sensitive markers for the differentiation state of articular primary chondrocytes. *Biochem J* **358**, 17-24 (2001).
63 Varas, L. *et al.* Alpha10 integrin expression is up-regulated on fibroblast growth factor-2-treated mesenchymal stem cells with improved chondrogenic differentiation potential. *Stem Cells Dev* **16**, 965-978, doi:10.1089/scd.2007.0049 (2007).

64 Tavella, S. *et al.* Regulated expression of fibronectin, laminin and related integrin receptors during the early chondrocyte differentiation. *J Cell Sci* **110 (Pt 18)**, 2261-2270 (1997).
65 Hirsch, M. S., Lunsford, L. E., Trinkaus-Randall, V. & Svoboda, K. K. Chondrocyte survival and differentiation in situ are integrin mediated. *Dev Dyn* **210**, 249-263, doi:10.1002/(SICI)1097-0177(199711)210:3<249::AID-AJA6>3.0.CO;2-G (1997).
66 Aszodi, A., Hunziker, E. B., Brakebusch, C. & Fässler, R. Beta1 integrins regulate chondrocyte rotation, G1 progression, and cytokinesis. *Genes Dev* **17**, 2465-2479, doi:10.1101/gad.277003 (2003).
67 Bengtsson, T. *et al.* Loss of alpha10beta1 integrin expression leads to moderate dysfunction of growth plate chondrocytes. *J Cell Sci* **118**, 929-936, doi:10.1242/jcs.01678 (2005).
68 Zemmyo, M., Meharra, E. J., Kühn, K., Creighton-Achermann, L. & Lotz, M. Accelerated, aging-dependent development of osteoarthritis in alpha1 integrin-deficient mice. *Arthritis Rheum* **48**, 2873-2880, doi:10.1002/art.11246 (2003).
69 Zhang, L. *et al.* Chondrogenic differentiation of human mesenchymal stem cells: a comparison between micromass and pellet culture systems. *Biotechnol Lett* **32**, 1339-1346, doi:10.1007/s10529-010-0293-x (2010).
70 Bosnakovski, D. *et al.* Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis. *Biotechnol Bioeng* **93**, 1152-1163, doi:10.1002/bit.20828 (2006).
71 Livak, K. J. S., T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408 (2001).
72 Schmittgen, T. D. L., K. J. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* **3**, 1101-1108 (2008).
73 Dorak, T. *Real-time PCR*. 1 edn, (Taylor & Francis, 2006).
74 Loeser, F. R. Chondrocyte integrin expression and function. *Biorheology* **37**, 109-116 (2000).
75 Goessler, U. R. *et al.* In vitro analysis of integrin expression during chondrogenic differentiation of mesenchymal stem cells and chondrocytes upon dedifferentiation in cell culture. *Int J Mol Med* **17**, 301-307 (2006).
76 Wang, L. *et al.* Cartilage oligomeric matrix protein maintains the contractile phenotype of vascular smooth muscle cells by interacting with alpha(7)beta(1) integrin. *Circ Res* **106**, 514-525 (2010).

Note

This project was accomplished by Amanda Verpoorte, Master student, with the help of Vanessa LaPointe, PhD student. The primer validation, cell culture work, RNA extraction, cDNA synthesis, and qPCR of the alpha integrin subunits were done by Amanda Verpoorte. The qPCR of the beta integrin subunits and phenotype markers presented in this report were done by Vanessa LaPointe.